

John Fernandes



COMPREHENSIVE BIOTECHNOLOGY

Comprehensive Biotechnology

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COMPREHENSIVE BIOTECHNOLOGY

by
John Fernandes



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Preface

Biotechnology has evolved as an ill-defined field from inter-related activities in the biological, chemical and engineering sciences. Inevitably, its literature is widely scattered among many specialist publications. There is an obvious need for a comprehensive treatment of the basic principles, methods and applications of biotechnology as an integrated multidisciplinary subject, 'Comprehensive Biotechnology' fulfills this need. It delineates and collates all aspects of the subjects and is intended to be the standard reference book in the field.

In the preparation of this book, the following conditions were imposed. (1) Because of the rapid advances in the field, it was decided that the book would be comprehensive but concise enough to enable completion within one book. (2) Because of the multidisciplinary nature of biotechnology, an experienced author of national repute was required. (3) Again, because of the multidisciplinary nature of work, it was virtually impossible to use a completely uniform system of nomenclature for symbols. However, IUPAC provided us provisional guidelines. (4) Since the work is intended to be useful to both beginners as well as veterans in the field, basic elementary material as well as advanced specialist aspects are covered (5) Because of space constraints, a value judgement was made of the relative importance of topics in terms of their actual rather than potential commercial significance. (6) Finally, a delicate balance of material was required in order to meet the objective of providing a comprehensive and stimulating coverage of important practical aspects as well as the intellectual appeal of the field.

Indeed, if our compilation helps to draw to the areas of basic science and engineering in the contributory disciplines that are most relevant to biotechnology, and to promote more research for

those areas, it will be serving a valuable purpose. In keeping with a narrowly utilitarian age, the fashion for ruthlessly target oriented research is rapidly leading to a situation in which empirical applications, based on superficial extensions of old understanding, have outstripped the basic knowledge that is needed. Today's shiny edifice of biotechnology is in serious danger of collapsing into inadequate foundations, and the numerous national and international commissions and committees that exists to 'promote' biotechnology need to have their attention.

This, then, is our plea in mitigation to those who will use our book to make their entry into biotechnology. The enthusiasts who are already there will have different objections; there is little here for flavour-of-the-month fans. By describing a broad selection of process biotechnology as it is, rather than speculating about what it might become, we hope our readers will emerge better equipped to understand not only what they are doing today, but what they might do tomorrow.

Biotechnology is-or it can be-clean technology, green technology and human-scale technology. It lends more rapidly to improving the human condition than to terminating it. It will become our major technology if mankind has any future, and today we are only seeing its very beginnings. But even in today's world it can also make good brassnosed economics-if that is all you care about.

'Comprehensive Biotechnology' is aimed at a wide range of user needs. Student's, teachers, researchers and others in academic, industry and government are addressed. The requirements of the following groups have been given particular consideration: (1) chemists, specially biochemists, who require information on the chemical characteristics of enzymes, metabolic processes, products and raw materials, and on the basic mechanisms and analytical techniques involved in biotechnological transformations; (2) biologists, especially microbiologists and molecular biologists, who require information on the biological characteristics of living organisms involved in biotechnology and the development of new life forms by genetic engineering techniques; (3) health scientists, especially nutritionists and toxicologists, who require information on biohazards and containment techniques, and on the quality of products and by-products of biotechnological processes, including the pharmaceuticals, food and beverage industry; (4) chemical

engineers, especially biochemical engineers, who require information on mass and energy balances and rates of processes, including fermentations, product recovery and feedstock pretreatment, and the equipment for carrying out these process; (5) civil engineers, especially environmental engineers, who require information on biological waste treatment methods and equipment, and on contamination potentials of the air, water and land within the ecosystem, be industrial and domestic effluents; (6) other engineers, especially agricultural and biomedical engineers, who require information on advances in the relevant sciences that could significantly affect the future practice of their professions.

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Ethanol

The preparation of distilled alcohol spirits was first described in the 12th century with the first reported (nonbeverage) uses of spirits as an incendiary, a solvent and later medicinally.

The availability after World War II of cheap ethylene (as a by-product of natural gas recovery and of gasoline production) led to the rapid growth of the synthetic ethanol industry based on the esterification-hydrolysis of ethylene in concentrated sulfuric acid-water solution.

Raw Materials Selection and Preparation

Raw materials for fermentative ethanol production can be divided into three classifications by carbohydrate type: saccharine materials, starchy materials and cellulose.

Saccharine Materials

Major saccharine raw materials are sugar cane or sugar beet juice, high test molasses, black-strap molasses, fruit pulp and juice wastes, cane sorghum and they have alternate use as cattle feed components, and their prices are set primarily based upon feed value and shipping costs. Sugar cane and sugar beets are used for edible sugar production and this sets their market value. Prices for all of these commodities are subject to large fluctuations.

Sugar cane juice

Owing to transportation cost, the direct use of sugar cane is limited to alcohol plants located within farming districts. In India 53% of alcohol plants process cane directly as raw material.

Sugar cane juice is recovered by a milling train consisting of knife cutters followed by a pounding mill to rupture the cell walls and 3–5 high-pressure roller presses to express the juice. Crushed

cane is hot water rinsed to aid sugar extraction between pressing cycles. Total water used is 25% of the weight of the cane and 85-90% of the fermentable sugars are extracted. The resulting juice is 12-16% sucrose sugar. The yield of sugar from cane is typically 125 kg per metric ton. A mascerated solid bagasse residue (50% moisture) remains after sugar expression, and this can be used as a boiler fuel, resulting in a considerable economy in distillation and stillage evaporation fuel. Two kg of bagasse (dry weight), with a heating value of 19 700 kJ kg⁻¹ (8500 Btu lb⁻¹), are produced for every kg of sugar. Sugar cane wax and aconitic acid can also be recovered as by-products. Cane crushers are high-pressure steam driven using from 1.0-1.2 kg steam per kg sugar recovered. Low-pressure exhaust steam from the crushers is reused for distillation.

Sugar beet juice

Direct use of sugar beets requires location of the alcohol plant within the farming district. Unlike sugar cane, however, sugar beet production is not limited to tropical and subtropical climates. A large sugar beet alcohol industry existed in France in the 1940s.

Beet sugar is expressed by first slicing beets into V-shaped cossette wedges, followed by sugar extraction in continuous counter-current hot water (85°C) diffusers to produce a 10-15 wt% sugar solution. The cellulosic beet pulp residue is generally used as a low-grade cattle feed roughage.

High test molasses

High test molasses is a concentrated sugar solution, allowing easy shipping and long-term storage. Sugar cane or beet juice is first partially hydrolyzed with dilute acid to convert sucrose to noncrystallizable invert sugar monomers (glucose and fructose). The juice is then concentrated to 70-78 wt% sugar. Table 1.1 gives a typical composition of high test molasses. Owing to thermal decomposition of sugars during evaporation, and to the presence of small concentrations of yeast inhibitors, high test molasses sugars are generally only 95% fermentable.

Blackstrap molasses

Blackstrap molasses is the noncrystallizable residue by-product of table sugar manufacture. The sugar juice is first treated with lime (0.75 kg per metric ton cane) at 100 °C to neutralize organic acids.

On cooling, salts, coagulated albumen, fats and gums are precipitated. The clarified juice is concentrated to 60 wt% solids by multieffect evaporation and sucrose is precipitated by further evaporation in vacuum pans. After three or four evaporations, high viscosity prevents further crystallization. The sugars are primarily sucrose and non-crystallizable invert mixed with the now concentrated inorganic salts and soluble nonsugar organic residues retained from the juice or generated during evaporation (Table 1.1). Approximately 27 kg of blackstrap are obtained from every metric ton of cane processed and 2.5 l of molasses (3.5 kg) is required to produce one liter of 95 wt% ethanol.

Table 1.1 : Composition of High Test and Blackstrap Molasses

	<i>High test molasses</i>	<i>Blackstrap molasses</i>
Total solids	80-85%	83-85%
Invert sugar	40-60%	12-18%
Sucrose	35-15%	40-30%
Fermentable sugar	70-78%	50-55%
Organic nonsugars	4-8%	20-25%
Ash	2-4%	7-10%

Up to 17% of blackstrap molasses is nonfermentable reducing compounds resulting from high-temperature destruction of sugars during evaporation. Fructose undergoes dehydration and reduction to 1, 3-fructopyranose, and both glucose and fructose undergo salt-catalyzed condensation with cane amino acids, resulting in dark nonfermentable caramel residues. Sugars are also decomposed to volatile hydroxymethylfurfural, acetoin, formic and levulinic acid. Vapor-recompression vacuum sugar evaporation reduces evaporation temperatures and can thus reduce sugar decomposition and substantially increase the yield of ethanol from blackstrap molasses. Flash cooling of molasses after high-temperature evaporation can also reduce sugar decomposition and increase the ethanol yield.

In blackstrap molasses, components which are inhibitory to yeast (Primarily calcium salts from the lime treatment and organic decomposition compounds) are quite concentrated due to the repeated crystallizations. These reduce final alcohol yield and typically only 90% of untreated blackstrap molasses sugar is utilizable. In the Arroyo process (Arroyo, 1949) ammonium sulfate

and calcium superphosphate are added to concentrated molasses, which is heated to 80 °C with the pH adjusted to 4.5-5.2. A sludge, containing calcium sulfate and organic inhibitors in colloidal form, settles from the solution and the clarified molasses is then used in fermentation. In the Reich process, concentrated molasses is heated to 70-90 °C and sulfuric acid is added to adjust the pH to 3-4. Again, a sludge is removed and the clarified solution used for fermentation.

Unfortunately, both of these methods also result in the precipitation of natural yeast growth factors present in the raw molasses, and additional nutrients must be added to clarified molasses for rapid yeast growth. Some sugar decomposition also takes place at the elevated clarification temperature. Blackstrap molasses clarification is not necessary for simple batch fermentation, but is essential if yeast or stillage is to be recycled. In addition, molasses clarification can be beneficial in reducing scale formation (from calcium salt deposition) in distillation and stillage evaporation heat-transfer equipment.

Inhibitor concentrations are much lower in high test molasses and cane juices, but these may also be pretreated to further increase yield and eliminate scaling problems.

Sweet sorghum

Sweet sorghum is a tall grass with high sucrose content in the stems. It is widely grown for cattle forage. Sweet sorghum has received much attention because it can be readily cultivated under a wide variety of growth conditions and has a yield of sugar per hectare slightly higher than that found for sugar beets. After sugar expression, the cellulosic sorghum residue can be used as a cattle feed roughage source.

Fruits and juices

Fruit cannery wastes can be used as a substrate for alcohol production. After orange, apple and pineapple juices have been squeezed, additional sugars can be extracted from the pulp by hot water diffusion. Pineapple canning yields excess juice, and in Hawaii up to 1.25 million liters per year of alcohol has been produced from this waste with 15-25 liters of juice required per liter of alcohol product. Ion exchange processes now allow the recovery of sugar from very dilute waste streams, and dilute cannery wastes could be used as an alcohol source while reducing the BOD of

cannery effluents. Production from this source will, of course, be limited, but does well illustrate the potential to integrate alcohol production into a waste disposal process, thereby producing a valuable product at very low raw material cost.

Whey

Whey, produced as a by-product of cheese manufacture, is high (4.5-5.0%) in lactose. Lactose is a disaccharide composed of a glucose and a galactose unit and is not fermentable by most yeasts. The strains *Torula cremoris* and *Candida pseudotropicalis* readily ferment lactose to ethanol and this process has been used at many small installations since the 1940s. Ethanol production from whey is limited and must compete with use for whey in cattle feed and antibiotic production.

Starchy Materials

Starchy raw materials include cereal grains, starch root plants and some cacti. The grains and root plants are all used both for human consumption and animal feed. Prices are set based on these uses and fluctuate widely based on annual crop yields and international demand.

The carbohydrate in the starchy plants is not directly fermentable by yeast and these materials must be pretreated to hydrolyze the starch to simple sugars. Starch is first hydrated and gelatinized by milling and cooking, and then broken down to fermentable sugars by diastatic enzymes or weak acids. Capital investment for starchy material feed pretreatment can typically make up 15-20% of the total alcohol plant cost.

Cereal grains

Important cereal grains are corn, wheat, rice, barley and grain sorghum. Cereal grains are generally 50-65% starch and as high as 80% for rice. Corn is the major grain used for alcoholic fermentation.

Grain processing has traditionally been by batch methods, but these are now often replaced by fully continuous processes. The grain is first air classified to remove dirt and cracked hulls. It is then ground (typically to 20-60 mesh) to allow easy wetting and then gelatinized by cooking.

Traditional atmospheric pressure processes have been almost entirely replaced by pressure cooking. The higher temperature

cooking allows reduced cooking time with less sugar degradation and 3-5% higher final yields. A coarser grind is allowable. Pressure cooking sterilizes the mash. Batch pressure cooking is by steam injection at typically 690 kPa (100 p.s.i.), into horizontal pressure vessels of up to 40 000 liter capacity with horizontal rake agitators. The mash, with a grain concentration of roughly 0.27 kg per liter of water and pH adjusted to 5.5, is heated to 135-150 °C and held for 10-30 minutes. Rapid cooling by pressure blowdown and then vacuum evaporation reduces the temperature to below 65 °C for the addition of enzymes. Blowdown steam may be recovered for other uses.

In the continuous cooking process, milled grain is first slurried with water in a stirred tank with a 1-5 min residence time. The slurry, again at roughly 0.27 kg grain per liter of water and pH 5-6, is continuously metered through a steam-jet heater into a cooking tube with a plug flow residence time of less than 5 min and at up to 180 °C cooking temperature. Flash cooling is used to reduce rapidly the mash temperature and halt sugar breakdown. The continuous cooking process gives more uniform cooking at lower steam consumption and capital investment than the batch process.

Traditional starch hydrolysis has been conducted using barley malt. Sprouted barley contains a high diastase activity. Ground dried barley malt is reactivated by slurrying with warm water (0.19 kg l⁻¹). After 5 min the malt solution is mixed with cooled mash to achieve a ratio of typically 1 part malt to 10 parts other grain at 63°C. Grain starch is composed of straight-chained amylose and branched amylopectin. The amylose is readily hydrolyzed to maltose. Amylopectin is broken down partly to maltose, but largely to branched nonfermentable dextrans. After 40 min, 75-80% of grain starch is converted to maltose, the remainder to limit dextrans. The mash is then fermented, with starch hydrolysis continuing. The conversion of maltose to ethanol during fermentation favors further breakdown of dextrans to maltose. Fermentation pH must be kept above 4.1 to maintain enzyme activity. For conventional grain fermentations this final dextrin conversion, not the ethanol fermentation itself, becomes the rate limiting step in fermentation.

The continuous flow hydrolysis process is used in combination with continuous cooking (Fig. 1.1). A reactivated malt solution is continuously metered into the stream of cooked, cooled mash at 63°C in a plug flow pipe reactor with a 2 min residence time. The

mash is then further cooled to fermentation temperature. The conversion of starch to maltose is reduced to 70%, but the abbreviated time results in reduced denaturation of the diastatic enzymes. Conversion of residual dextrins in the fermenter is faster and more complete with a 2% final alcohol yield increase typical.

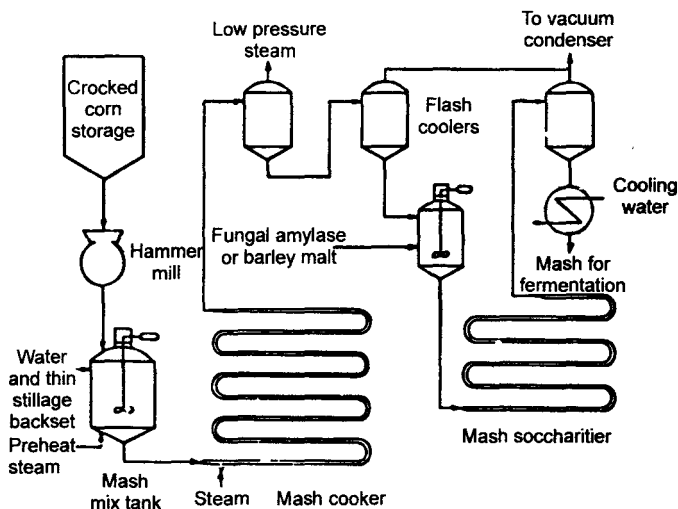


Fig. 1.1 : Continuous flow grain cooking and saccharification

An alternative to barley malt is mold bran. *Aspergillus oryzae* is grown on moist sprouting bran and produces amylase enzymes. The moldy bran can then be treated exactly as barley malt in carrying out the hydrolysis. Under some conditions, mold bran can be less expensive than barley malt and 2% final alcohol yield increases are typical.

Barley or bran can at no time be heated above 63 °C or the malt enzymes will be destroyed.

Thus sterilization is impossible, and a major disadvantage of malt and mold bran hydrolysis is the increased occurrence of fermenter contamination. Lactic acid and other bacteria introduced in the malt can reduce alcohol yield by 20%.

Roots, tubers and cacti

Roots, tubers and cacti can all be very high in carbohydrate content, giving final alcohol yields per hectare higher than the cereal grains. High water content makes storage difficult and

seasonal operation is required without costly drying to prevent spoilage. The protein content of these raw materials is low so that by-product recovery is not feasible and substantial by-product credits are not available to offset the raw materials costs. Materials used for alcohol production include potatoes, sweet potatoes, Jerusalem artichoke, manioc and sotol.

Potatoes may be 15-20% starch with alcohol yields of 4700 liters per hectare (476 gal per acre). Sweet potatoes, with a starch content as high as 30%, can have yields of up to 7200 l ha⁻¹. Potatoes were used extensively for industrial alcohol production in pre-World War II Germany under government price supports. 184 000 metric tons were used in the US in 1947 during a grain shortage. Except for such special circumstances, potato costs are generally prohibitive for ethanol manufacture.

Jerusalem artichoke (wild sunflower) tuber and manioc (tapioca, cassava) roots are high in inulin (a plant starch). Jerusalem artichokes have been used for industrial alcohol production in France. Manioc growth is limited to tropical climates, but yields as high as 190001 ha⁻¹ have been achieved 9% of Brazilian alcohol manufacture is now from manioc. Prussic acid (a yeast inhibitor) content is high in manioc, but this can be broken down by sun drying the kibbled root on the fields.

Dasyliion cactus (sotol) is a wild desert plant of interest as it would be harvested from otherwise unutilized lands. Dasyliion was harvested in Texas in 1944 for alcohol manufacture with a yield of up to 127 l t⁻¹.

Processing for the roots, tubers and cacti are all similar. After water rinsing, the materials are cut or chipped and then hammer milled to a mash. Processing is then similar to the steps for grain cooking and hydrolysis. An exception is that special inulase enzymes are used for the hydrolysis of Jerusalem artichoke or manioc inulin to fructose. For soft materials like potatoes and sweet potatoes, mashing and cooking may be partly combined in the Henze cooker. Whole or chopped substrate is batch loaded into a vertical pressure cooker. After cooking, the mash is blown down through the conical vessel bottom into a lower-pressure drop tub with violent boiling resulting. This 'steam explosion' disintegrates the substrate, thus eliminating the need for milling. Conventional hydrolysis processes are then used.

Cellulosic Raw Materials

Compositions of several proposed cellulosic feedstock are summarized in Table 1.2. These materials are soft woods or woody agricultural residues and are made up of cellulose, hemicellulose and lignin bound into a structure which gives a plant its rigidity and support. Cellulose polymer can be hydrolyzed to its glucose monomer units for fermentation to ethanol.

Table 1.2 : Composition of Cellulosic Raw Materials for Ethanol Production

	<i>Corn stover</i>	<i>Wheat straw</i>	<i>Rice straw</i>	<i>Rice hulls</i>	<i>Baga- sse fiber</i>	<i>Cotton gin trash</i>	<i>News print</i>	<i>Populus tristis</i>	<i>Doug- las fir</i>
Carbohydrate									
(% sugar equivalent)									
Glucose	39.0	36.6	41.0	36.1	38.1	20.0	64.4	40.0	50.0
Mannose	0.3	0.8	1.8	3.0	NA	2.1	16.6	8.0	12.0
Galactose	0.8	2.4	0.4	0.1	1.1	0.1	NA	NA	1.3
Xylose	14.8	19.2	14.8	14.0	23.3	4.6	4.6	13.0	3.4
Arabinose	3.2	2.4	4.5	2.6	2.5	2.3	0.5	2.0	1.1
Non-carbohydrate									
(%)									
Lignin	15.1	14.5	9.9	19.4	18.4	17.6	21.0	20.0	28.3
Ash	4.3	9.6	12.4	20.1	2.8	14.8	0.4	1.0	0.2
Protein	4.0	3.0	NA	NA	3.0	3.0	NA	NA	NA

For every 1 kg of grain harvested, 1-1.5 kg of straw, cobs, stover or other residue are generated. Only one-third of tree biomass is recovered as finished lumber. Cellulosics have received great attention as possible feedstocks on the assumption that raw material costs for agricultural and lumber waste materials should be negligible. Special cases do exist where the residues are collected as part of harvesting, but in these cases residues are already put to valuable use. Corn cobs and beet pulp, for instance, are used as animal feed. Sugar cane bagasse, sawdust and wood chips are used for process fuel or as fiberboard components.

Cellulosic paper wastes can be separated from municipal garbage by air flotation, but in this case the careless disposal of a pesticide or other toxic chemical by a single household could contaminate the material and force a plant shutdown. Intensive tree

farming has been proposed and studies suggest that fast growing popular varieties could be grown with a delivered dry wood price, but this has never been substantiated by an industrial trial.

Cellulose is far more chemically stable than starch and harsher acid hydrolysis conditions or more potent enzyme solutions are required for hydrolysis. Extensive pretreatment is often required to increase the accessibility of the cellulose which is otherwise well protected in its natural lignocellulosic structure. The cost of cellulose conversion to sugar is far higher than the cost of starch conversion. Yields are typically also much lower, requiring more raw materials and resulting in substantial waste streams requiring costly disposal.

A special case is sulfite waste liquor, which is the residue of partial wood hydrolysis from paper pulp production. In this case a partial acid hydrolysis is carried out to prepare a valuable bleached cellulose pulp and a dilute sugar solution is produced as an unwanted by-product. After pretreatment to neutralize yeast toxins, this waste can be fermented to produce alcohol. This process must compete, however, with aerobic yeast feed production from the sulfite liquor which requires less capital investment and is more effective in reducing the BOD of the waste liquor in preparation for discharge.

Few by-products of cellulose hydrolysis have proven high value, but there are promising possibilities. Lignin with a heating value of $29\,500\text{ kJ kg}^{-1}$ ($12\,700\text{ Btu lb}^{-1}$) is readily utilized as a boiler fuel. Lignin is a complex three-dimensional polymer of phenolic origin. Lignin can be used as a source of vanillin and syringic aldehydes, as an extender in phenolic plastics and as a lignin-formaldehyde binder for particle board, replacing phenol-formaldehyde. Further development of high-value applications for lignin and other by-products could go a long way toward offsetting the high cost of cellulose hydrolysis.

Slow acid hydrolysis

Twenty-one alcohol plants utilizing wood were operated prior to or during World War II. These plants used either the Scholler dilute (0.2–1.0%) sulfuric acid process or the Bergius concentrated (40–45%) hydrochloric acid process for wood hydrolysis. Yields for both processes were similar with up to 500 kg of sugar (70% fermentable glucose) produced per metric ton of coniferous sawdust

or bark-free chips. The Scholler process was less capital intensive and was therefore preferred. Wood chips were charged into Herculoy lined, acid-resistant pressure vessels (14 ton wood capacity). Dilute acid of 0.5-0.6% concentration was percolated down through the packed bed with continuous removal of sugar solution at the base. Reactors were heated by direct steam injection beginning at 350 kPa (50 p.s.i.) and increasing to 1135 kPa to hydrolyze the more resistant materials remaining at the end of a run. Total hydrolysis time was 2.5-3.5 hours. After removal from the vessel, the sugar solution was immediately neutralized with lime and flash cooled to minimize sugar decomposition caused by the harsh acid conditions. The resulting sugar solution is only 5-6 wt% sugars, 80% fermentable by yeast, and fermentation produces dilute alcohol solutions that are costly to distill. Despite the careful control of temperature and rapid flash cooling of product solutions, 40% of total sugars and 35% of fermentable product glucose are not recovered or are decomposed further by the acid to unwanted by-products such as furfural, methanol and sugar acids (especially levulinic). In addition to the loss of yield, these by-products are inhibitory to the yeast fermentation. After hydrolysis, the reactors were blown down and the lignin residue was filter pressed to 50% moisture and burned to help provide the 500 000 kg d⁻¹ (5 kg kg⁻¹ sugar) steam requirement of the plant. Chemical requirements for the plant were 0.65 kg sulfuric acid and 0.50 kg lime per kg of fermentable sugar produced.

Rapid acid hydrolysis

Careful kinetic studies have shown that high conversions of cellulose to sugar can be achieved and sugar degradation minimized by reacting at high temperature and pressure for very short periods. Based on these results, Rugg at New York University has developed and tested, at one ton cellulose per day pilot-plant scale, a continuous fast acid hydrolysis reactor. A twin corotating screw extruder (of the type used in plastics compounding and extrusion) is used. Hydropulped newspaper or sawdust is cram fed into the extruder which shears the material into a slurry and compresses it to a solid plug at 3450 kPa pressure, dewatering it to a cellulose concentration of 50%. High-pressure superheated steam is injected, raising the temperature to 240 °C. Near the extruder barrel outlet, 0.5% sulfuric acid is injected. The hydrolysis takes place with up to 60% sugar conversion in 20 seconds before the

plug is expelled from the screw through a high-pressure valve. The brown paste slug, containing up to 30% glucose, is flash cooled to prevent decomposition and the sugars are extracted in a two-stage countercurrent washer. For each kg of sugar produced, 2.1 kg of steam for heating and 0.75 kWh of electricity to drive the screw reactor are consumed. The solid residue can be burned to provide all required process steam.

Production of concentrated sugar solutions overcomes the problem of high distillation and evaporation costs associated with the slow acid hydrolysis process. Experiments are necessary to determine whether further treatment of the sugar solutions is necessary to remove inhibitors before fermentation. Plants to build a 50 ton per day demonstration plant have not been carried through.

Enzymatic hydrolysis of cellulotics

Enzymatic hydrolysis has the potential to overcome many of the drawbacks of acid hydrolysis. The conversion is carried out at ambient temperature and pressure, thus greatly reducing the cost of hydrolysis equipment. Sugar decomposition is eliminated, thus eliminating this cause for loss in yield and producing clean sugar streams for further processing. Costly neutralization and purification equipment is unnecessary, and hard to dispose of waste streams from acid neutralization are eliminated. Balancing these potential savings, extensive pretreatment to break down lignin and increase cellulose accessibility is required to achieve good yields and the cost of high activity cellulolytic enzyme solutions is at present very high.

For untreated substrates, cellulose is often present in a crystalline form which limits accessibility and defies enzymatic hydrolysis except for slow end cleavage. The cellulose is also protected by a lignin sheath inert to cellulolytic enzymes. Without pretreatment, sugar conversion from natural feedstocks is generally limited to less than 25%. Pretreatment processes are necessary to convert cellulose to an accessible amorphous form for rapid hydrolysis.

Ball or hammer milling reduces the degree of polymerization of both cellulose and lignin and reduces cellulose crystallinity. Sugar conversion can be increased to 60-70% by milling to -200 mesh, but milling is very energy and capital intensive. Partial delignification can be achieved by extraction with hot alcohol solutions (butanol

and higher) and the lignin recovered in a thermoplastic form. Cellulose crystallinity can be reduced by chemically swelling the polymer with alkali, ammonia or nitric oxide to break interstrand hydrogen bonds. These treatments can also remove a portion of the lignin. Treatment with ozone or cadoxen reduces the degree of polymerization of cellulose, decreasing crystallinity and increasing solubility. All of these chemical processes are costly, and extensive chemical recovery or waste disposal provisions are necessary just as in the direct acid hydrolysis processes.

Sulfite waste liquor

Alcohol is currently produced from sulfite pulping wastes at several large mills in Europe and the US. The sulfite pulping process is a treatment to remove hemicellulose and delignify wood to produce a clean cellulose fiber pulp for paper or rayon manufacture. Wood chips are pressure cooked at 140 °C with an aqueous solution of sulfurous acid containing calcium, ammonium or magnesium cation to provide 5-7% free sulfur dioxide. The dilute acid hydrolyzes hemicellulose almost completely to its monomer pentose sugars and renders lignin soluble by sulfonation. The cations neutralize the strong lignosulfuric acids to prevent excessive degradation of the cellulose. Amorphous cellulose, however, is hydrolyzed to glucose which can be fermented. After blowing down to atmospheric pressure, the liquor is screened or pressed from the cellulose fibers and the fibers are then washed in countercurrent flow. The resulting sulfite waste liquors contain 8-12% dissolved solids-organic decomposition products equal to almost half the weight of the original wood, in addition to the inorganic pulping chemicals. Sugars make up typically 20-30% of the dissolved solids. The proportion of fermentable hexose sugars is highest for soft woods and harsh pulping conditions and can reach up to 70%. For each metric ton of finished pulp produced, 4000-6000 l of waste liquor is generated.

Free and loosely combined sulfur dioxide is present at 5-15 g l⁻¹. Sulfur dioxide in low concentrations reduces ethanol yield by increasing acetaldehyde production. At the high concentrations present in raw waste liquor, it is completely toxic to yeast. Early plants (1907-1945) used direct addition of lime to neutralize sulfurous acid and simultaneously precipitate a portion of the lignosulfonic acids to detoxify the liquor for fermentation. Modern plants steam strip the liquor with 0.06 kg steam per l of liquor in a

roughly 20-stage column (Walker and Morgen, 1946). 10 kg of sulfur for recycle to the pulp digesters is recovered per metric ton of liquor and this offsets the steam cost.

A final lime neutralization (with approximately 0.4 kg lime per 1000 l liquor) is used to precipitate organics and adjust the pH.

A major drawback to the use of sulfite waste liquor is the low concentration of fermentable sugars—only 2-3 wt%. Evaporation prior to fermentation is impractical due to salt deposition problems in heat transfer equipment. Sugar bearing waste liquor can be recycled to the digesters to increase the sugar concentration of the subsequent liquor recovered. Up to 55 g l⁻¹ of sugar have been obtained in small scale experiments, but sugar degradation to sugar sulfonic acids and bisulfite addition compounds is increased. Molasses has been added to sulfite liquor in one US plant to increase the final alcohol product concentration and simplify distillation. If none of these methods are employed, then very dilute 1-1.5 wt% ethanol solutions are obtained from fermentation and special distillation methods must be employed to prevent extremely high separation energy requirements. Distillation is also complicated by the presence of increased levels of fermentation by-products—fusel oils, borneol, limonene, camphene and organic acids (McCarthy, 1954). Depending on the initial pulping conditions, 65-105 liters of ethanol can be produced per metric ton (15-25 gal per US ton) of pulp manufactured.

An important aspect of ethanol production from sulfite waste liquor is its role in waste disposal. Alcohol production reduces the liquor biological oxygen demand by 45-50%. Growth of cattle feed yeast competes with use of the sulfite liquor for ethanol manufacture. Aerobically grown *Torula* and *Candida* feed yeast can consume acetic acid and pentose sugars as well as hexose sugars; 95% of all reducing sugars are typically consumed with a 60% overall reduction in BOD.

Fermentation

Depending on design, fermentation equipment makes up 10-25% of the total fixed capital cost of an ethanol plant. Further, the fermentation process, by dictating feed sugar concentration and product cell and ethanol concentrations, specifies the major flows and concentrations throughout the remainder of the plant. The fermentation process, thus, is central to the overall plant design.

Organisms for Ethanol Fermentation

Organism alternatives

Yeasts are the only organisms currently used for large-scale industrial ethanol production. Yeasts produce ethanol with very high selectivity (only traces of by-products), are very hardy and are large compared with bacteria (allowing simplified handling).

Clostridium thermosaccharolyticum, *Thermoanaerobacter ethanolicus* and other thermophilic bacteria as well as *Pachysolen tannophilus* yeast are under intensive study for use in fermenting pentose sugars which are nonfermentable by ordinary yeast. These bacteria also convert hexose sugars and have been considered as an alternative to yeast since very high-temperature reactions would allow simple continuous stripping of ethanol product from the active fermenting mixture, thus eliminating end-product inhibition effects. Organisms so far studied, however, produce excessive quantities of undesirable by-products or are limited to producing only dilute ethanol beers. The bacteria also require strict anaerobic conditions which would be difficult to maintain on an industrial scale.

The bacterium *Zymomonas mobilis* is also under intensive study. This organism ferments glucose to ethanol with a typical yield 5-10% higher than for most yeasts, but is less ethanol tolerant than industrial yeast strains. The small bacterium is also difficult to centrifuge. An important possibility for the future is the development of an organism especially tuned to rapid ethanol production. *Zymomonas* is a simple procaryote and, hence, is more amenable than yeast to genetic modification. Attempts are under way to increase the ethanol tolerance of *Zymomonas*, to allow utilization of pentose sugars and to impart flocculent characteristics for improved centrifugability.

Yeast strain selection

Yeast strains are generally chosen from among *Saccharomyces cerevisiae*, *S. ellypsoideus*, *S. carlsbergensis*, *S. fragilis* and *Schizosaccharomyces pombe*. For whey fermentation, *Torula cremoris* or *Candida pseudotropicalis* is used.

Yeasts are carefully selected for: (1) high growth and fermentation rate; (2) high ethanol yield; (3) ethanol and glucose tolerance; (4) osmotolerance; (5) low pH fermentation optimum; (6)

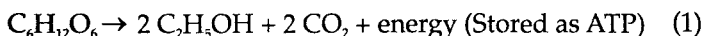
high temperature fermentation optimum; (7) general hardiness under physical and chemical stress. High growth and fermentation rate allows the use of smaller fermentation equipment. Ethanol and glucose tolerance allows the conversion of concentrated feeds to concentrated products, reducing energy requirements for distillation and stillage handling. Osmotolerance allows the handling of relatively 'dirty' raw materials such as blackstrap molasses with its high salt content. Osmotolerance also allows the recycle of a large portion of stillage liquids, thus reducing stillage handling costs. Low pH fermentation combats contamination by competing organisms. High temperature tolerance simplifies fermenter cooling. General hardiness allows yeast to survive both the ordinary stress of handling (such as centrifugation) as well as the stresses arising from a plant upset.

The years of careful selection by industrial use have led to yeast strains with these desirable characteristics. Many of the best strains are proprietary, but others are available from the culture collections.

Fermentation Kinetics

Yeast metabolic pathways

In the anaerobic pathway, glucose is converted to ethanol and carbon dioxide *via* glycolysis. The overall reaction (equation 1) produces two moles of ethanol and carbon dioxide for every mole of glucose consumed, with the reaction energy stored as two moles of ATP for use in biosynthesis or maintenance.



Via this pathway, every gram of glucose converted will yield 0.511 g of ethanol. Secondary reactions consume a small portion of the glucose feed, however, to produce biomass and secondary products and Pasteur found that the actual yield of ethanol from fermentation by yeast is reduced to 95% of the theoretical maximum (Table 1.3). When complex substrates, typical of industrial practice, are used, further by-products are generated and the ethanol yield is reduced typically to only 90% of the theoretical (0.46 g g⁻¹ glucose).

Via aerobic metabolism, sugar is converted completely to carbon dioxide, cell mass and by-products, with no ethanol formed, and aerobic metabolism must be avoided.

Table 1.3 : Optimum Yields from Anaerobic Fermentation by Yeast

<i>Product</i>	<i>g per 100 g glucose</i>
Ethanol	48.4
Carbon dioxide	46.6
Glycerol	3.3
Succinic acid	0.6
Cell mass	1.2

Effect of sugar concentration

Hexose sugar (glucose, fructose, galactose or maltose) is the primary reactant in the yeast metabolism. Under fermentative conditions, the rate of ethanol production is related to the available sugar concentrations.

$$v = v_{\max} C_s / (K_s + C_s) \quad (2)$$

At very low substrate concentrations (below about 3 g l⁻¹), the yeast is starved and productivity decreases. At higher concentrations a saturation limit is reached so that the rate of ethanol production per cell is essentially at its maximum up to 150 g l⁻¹ sugar concentration. Above 150 g l⁻¹, catabolite (sugar) inhibition of enzymes in the fermentative pathway becomes important, and the conversion rate is slowed.

An important secondary effect of sugar is catabolite repression of the oxidative pathways (the Crabtree effect). At above 3-30 g l⁻¹ sugar concentration (depending on yeast strain), the production of oxidative enzymes is inhibited, thus forcing fermentative metabolism. This catabolite repression is not found in all yeasts and is a desirable property which is selected in industrial strains.

Effect of ethanol

Ethanol is toxic to yeast and high ethanol tolerance is a desirable trait selected for in industrial strains. Ethanol inhibition is directly related to the inhibition and denaturation of important glycolytic enzymes, as well as to modification of the cell membrane.

Effect of oxygen

It is important to avoid a high degree of aerobic metabolism which utilizes sugar substrate but produces no ethanol. It has been

found, however, that trace amounts of oxygen may greatly stimulate yeast fermentation. Oxygen is required for yeast growth as a building block for the biosynthesis of polyunsaturated fats and lipids required in mitochondria and the plasma membrane. High sugar concentrations is adequate to repress aerobic sugar consumption in yeasts which show the Crabtree effect. For other yeasts or at low sugar concentrations, the oxygen supply should be limited. Trace amounts (0.7 mmHg oxygen tension) of oxygen are adequate and do not promote aerobic metabolism.

Effect of pH

Fermentation rate is sensitive to pH, but most distiller's yeasts show a broad pH optimum from at least pH 4-6. This range is lower than that for typical bacteria. Further, most yeasts can tolerate exposure to acid solutions of pH as low as 2 without permanent damage.

Effect of temperature

High temperature tolerance is a desirable characteristic selected for in distillery yeasts and most distillery yeasts have a temperature growth optimum of 30-35 °C. The optimum fermentation temperature at low alcohol concentrations is often slightly higher (up to 38 °C), but alcohol tolerance is improved at reduced temperatures. Exposure to temperatures above the optimum results in excessive enzyme degradation and loss of yeast viability. Yeast metabolism liberates 11.7 kcal of heat for each kilogram of substrate consumed.

Additional nutrient requirements

In addition to providing a sugar source for ethanol production, fermentation mash must also provide the secondary nutrients necessary for cell maintenance and growth. In laboratory tests, very rapid cell growth and ethanol production and high yields are achieved with a glucose medium supplemented with NH_4Cl , MgSO_4 , CaCl_2 and yeast extract. Ammonium ions provide nitrogen for protein and nucleic acid synthesis. Yeast extract is the water-soluble extract of autolyzed yeast and contains all necessary yeast growth factors: amino acids, purines, pyrimidines and vitamins as well as minerals. Phosphorus, potassium (from the yeast extract), magnesium and calcium are incorporated into cell mass and are also cofactors activating several enzymes.

To establish better the yeast nutritional requirements, worker developed a synthetic, totally defined medium (Table 1.4) which (after adaptation of the yeast in continuous culture) gives high productivity and yield. The minerals, $(\text{NH}_4)_2\text{SO}_4$ and KCl, and the vitamins, biotin and pantothenate, are stimulatory to growth, while thiamine and pyridoxine increase specific ethanol productivity. Thiamine may also increase ethanol tolerance.

Table 1.4 : Synthetic Medium for Yeast Fermentation

<i>Nutrient</i>	<i>Concentration</i>	<i>Nutrient</i>	<i>Concentration</i>
Glucose	100 g l ⁻¹	MnSO ₄	1.3 mg l ⁻¹
(NH ₄) ₂ SO ₄	3.54 g l ⁻¹	KI	0.5 mg l ⁻¹
KCl	0.625 g l ⁻¹	FeSO ₄	0.5 mg l ⁻¹
H ₃ PO ₄	0.411 g l ⁻¹	CoSO ₄	0.3 mg l ⁻¹
MgSO ₄	0.061 g l ⁻¹	Pyridoxine	3.33 mg l ⁻¹
CaCl ₂	0.022 g l ⁻¹	Pantothenate	2.00 mg l ⁻¹
H ₃ BO ₃	5.0 mg l ⁻¹	Thiamine	1.34 mg l ⁻¹
ZnSO ₄	2.8 mg l ⁻¹	Inositol	1.26 mg l ⁻¹
Al ₂ (SO ₄)	1.5 mg l ⁻¹	Biotin	5.25 mg l ⁻¹
CuSO ₄	1.3 mg l ⁻¹		

Secondary component inhibition

Yeast growth and ethanol production can be inhibited by fermentation by-products or by nonmetabolized feed components. These components become concentrated when backsetting is used and this limits the fraction of distiller's residue which can be recycled.

Acetate and lactate are the most important inhibitory fermentation by-products. Lactic acid and, to a lesser extent, acetic acid are partially removed in stripping and fermentation by-products do not normally establish the limit on backsetting.

Specific feeds may be high in certain inhibitors. Sulfite waste liquor may be high in sulfurous acid and furfural. Blackstrap molasses may contain high concentrations of calcium salts. High temperature sugar concentration and sterilization in the presence of salts (especially phosphates) and proteins can produce yeast toxins.

Fermentation Processes

Conventional batch fermentation

Batch fermentation begins with the production of an active yeast inoculum. This can be either by the conventional serial growth method or by the rapid semiaerobic method. Aseptic techniques are used throughout. In the serial growth method a pure culture inoculum from an agar slant is used to seed a laboratory shake flask. At the peak of growth (12-24 h) this culture is used to seed a succeeding culture 30-50 times larger. This is repeated, generally through three laboratory stages and two or three plant semiworks stages, to produce the final 2-5 vol% inoculum for the primary fermentation. The inoculum is grown on a medium similar to the final fermentation mash to minimize acclimatization time in the final fermenter, but higher levels of yeast growth nutrients may be used to produce a high cell density (typically 150 billion cells l^{-1}).

An inoculum 3-4 times more concentrated in yeast can be produced by the rapid semiaerobic method. Yeasts are grown in an aerated and agitated semiwork fermenter operated in fed batch mode. A large portion (20-25%) of the previous batch is retained to provide an inoculum. A high nutrient medium is added and pH and temperature are controlled. Sterile air is sparged at a rate of one-eighth volume of air per fermenter volume per minute. Aerobic metabolism is stimulated and a cell density of 500 billion cells l^{-1} is reached in 5h. This high cell density allows the use of a proportionately smaller inoculum to the final fermenter, and a smaller propagating fermenter can be used.

Cylindro-conical Nathan vessels are preferred for fermentation as these promote better circulation and allow through drainage for cleaning. For very large plants, sloped-bottom cone-roof tanks of up to 1 million liters volume are used and these large vessels are often agitated only by carbon dioxide evolution during fermentation.

After emptying and rinsing from the previous batch, mash at 13-17 wt% sugar is pumped to the fermenters. Once 20% full, the inoculum is added to allow growth during the remainder of the filling cycle, which can last 4-6 h.

Fermentation temperature is regulated by circulating cooling water through submerged coils, circulating the mash through external heat exchanges, or simply spraying the vessel walls with cool water (adequate for small fermenters only). The feed is generally

introduced at 25-30 °C, and the temperature allowed to gradually rise as heat is evolved. The temperature thus varies from 30-35 °C during the initial period (which is optimal for yeast growth). Cooling is then used to prevent the temperature from exceeding 35-38 °C, which is optimal for ethanol production. These temperatures may be modified depending on the yeast strain used.

Stillage backset provides excellent buffering. The pH is set initially at from 4.5-5.5 and decreases only slowly, generally holding at pH 4.0 or above. This is especially important for fermentation of grain mashes with simultaneous dextrin hydrolysis, as many amylase enzymes are rapidly denatured at lower pH.

After 20 hours a maximum in ethanol productivity is reached. The effects of reduced sugar concentration and ethanol inhibition then become important. The fermentation continues at a decreasing rate until, at 36 hours, 94% of the sugar is utilized and a final ethanol content of 69 g l⁻¹ is achieved. The average volumetric ethanol productivity over the course of the fermentation is 1.9 g l⁻¹ h⁻¹.

Fermentation time will vary depending on yeast strain and substrate. Hawaiian and Cuban blackstrap molasses fermentation usually requires 36h. Molasses from Java may require as long as 72 h. These times are reduced when molasses clarification is used. Grain fermentation requires 40-50h to allow complete residual dextrin conversion.

After fermentation, the beer is pumped to a beer well to provide a continuous feed to distillation. The fermenters are then cleaned and prepared for another cycle.

The fermentation rate can be increased 30-40% by improved agitation and temperature regulation. Turbine impellers have been used in smaller fermenters (100 000 l or less). For large fermenters, improved agitation and temperature control are achieved by rapidly circulating the beer through an external heat exchanger. The cooled beer is pumped back into the head of the fermenter tangentially and at a high velocity.

Contamination by lactic acid bacteria is occasionally a problem and alcohol yield can be reduced by as much as 20%. Such contamination is more likely when stillage back-set is used, allowing contaminating organisms to accumulate and acclimatize to the fermentation conditions. Aseptic operation, with complete sterilization of the very large mash volume, was considered

impractical until recently. Growth of organisms other than the seed yeast is generally restricted by the adverse conditions of low pH and high sugar or alcohol concentration, and the rapid growth of the yeast compared with contaminants was relied upon in place of aseptic techniques. Highly efficient continuous media sterilization now makes aseptic operation quite practical. The medium is heated by steam injection to 135-140 °C and held in plug flow for 1-2 min, resulting in essentially complete sterilization. Cooling is by flashing to regenerate steam or by heat exchange to preheat incoming feed. The steam requirement is only 3.5 kg steam per 100 kg mash. Small spherical head fermenter vessels can be sterilized by pressurizing with steam. For very large fermenters this is impractical and antiseptic solutions such as ammonium bifluoride, iodine, sodium hypochlorite or formalin are automatically sprayed to rinse the vessel walls before filling. Heat exchangers and transfer lines can be steamed.

A batch fermentation layout incorporating teamed heat exchange and chemical sterilization systems is shown in Figure 1.2.

The Mellé-Boinot fermentation process

The Melle-Boinot process achieves a reduced fermentation time and increased yield by recycling yeast. Cell density at the beginning of a conventional fermentation is quite low. Ethanol production rate

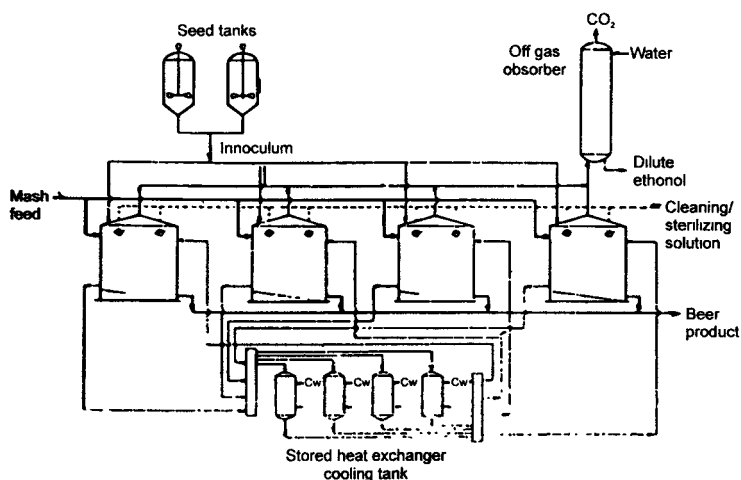


Fig. 1.2 : Batch fermentation equipment layout incorporating teamed heat exchange and chemical sterilization systems

is proportional to cell density and the initial growth phase of a conventional fermentation (lasting up to 15h) is thus relatively unproductive. In the Mellé-Boinot process, yeast cells from the previous fermentation are recovered by centrifugation and up to 80% are recycled. The initial cell density of a batch is thus as high as 80 billion cells l^{-1} and very rapid ('boiling') fermentation begins almost immediately. With the long growth phase eliminated, the overall fermentation cycle time is reduced by one-half to two-thirds, increasing volumetric productivity to typically $6 \text{ g l}^{-1} \text{ h}^{-1}$.

The Mellé-Boinot process was generally developed for and is widely used in sulfite waste liquor fermentation, where the low sugar concentrations require maximum yield and cell recycle achieves much higher cell densities and reduced fermentation time. The process is widely used in Europe for molasses fermentation.

Sugar clarification is essential, though, for good centrifuge performance and to maintain high yeast viability. Yeast recycle will reduce the fermentation time for grain fermentations only if the starch is preconverted to glucose by acid hydrolysis or high activity glucoamylase enzymes. Otherwise dextrin hydrolysis in the fermentation will still be limiting and little advantage gained.

Simple continuous flow fermentation

Continuous flow fermentation processes have been used in industrial sulfite waste liquor fermentation since the 1930s. The antiseptic qualities of sulfite liquor minimize the possibility of adverse contamination and allow long continuous runs without shutdowns for cleaning. Early attempts at continuous fermentation of molasses and grain hydrolyzates on an industrial scale were unsuccessful due to contamination problems and these plants were retrofit for batch operation. With continuous media sterilization and aseptic plant techniques, the contamination problem has been overcome, as is illustrated by the success of continuous molasses fermentation plants and many continuous beer brewing plants.

In laboratory tests with carefully optimized conditions of temperature, pH, agitation and flow rate, much higher productivities have been achieved. Molasses at $130 \text{ g sugar l}^{-1}$ has been fermented to completion in a 7 h residence time, corresponding to a volumetric productivity of $8.3 \text{ g l}^{-1} \text{ h}^{-1}$. A nutrient supplemented glucose medium of $130 \text{ g sugar l}^{-1}$ could be completely utilized in

9.5 h residence time. This fermentation was continued for 60 days without decline in productivity.

The Biostil process

The Biostil process (Fig. 1.3) is a modification of the continuous fermentation process with recycle, whereby the fermentation and distillation are closely coupled and a very high stillage backsetting rate is used. Fermenter beer is continually cycled (through a centrifuge for yeast recycle) to a small rectifying column where ethanol is removed. The majority of the ethanol depleted beer (with residual sugars and nonfermentables) is then recycled to the fermenters. The yeast cell density is maintained at 500 billion cells per liter. The fermenter ethanol concentration can be maintained at any desired non-inhibitory level by adjustment of the beer cycle rate. The large liquid recycle provides an internal dilution so that very concentrated feeds can be processed. Liquid flows external to the recycle loop are greatly reduced. Less water is consumed and a more concentrated stillage is produced. The flow rate capacities of most auxiliary equipment can be substantially reduced.

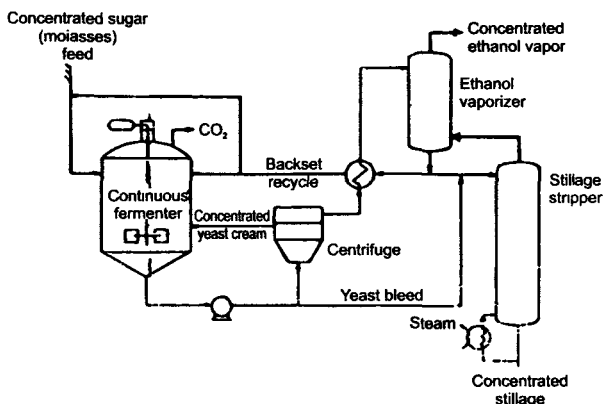


Fig. 1.3 : Biostil fermentation process

Extensive heat exchange is incorporated into the beer cycle loop to maintain energy efficiency. As bacterial are not well separated by the centrifuge, the cycling through a hot distillation stage also provides a continuous pasteurization and infection risk is reduced. As with the Mellé-Boinot process, ethanol yield is increased by cell recycling.

With ethanol inhibition overcome, the Biostil process is limited instead by the build-up of toxic nonfermentable feed components and fermentation by-products. In pilot plant studies the stillage flow could be reduced by a factor of 20 for concentrated cane syrup, but only a factor of 3 for blackstrap molasses with its high nonfermentable content. The process can also be applied to prehydrolyzed starch feeds.

Tower fermenter

An interesting fermenter arrangement which has been suggested for rapid industrial alcohol production but not yet used commercially is the APV tower fermenter. This system has been used commercially since 1965 for the production of beverage beers.

The fermenter consists of a cylindrical tower typically 2 m in diameter and 15 m tall and is topped by a larger diameter settling zone fitted with baffles. A 'sticky' flocculent yeast with very high settling rate is used. The prehydrolyzed sugar wort is pumped into the base of the tower and reaction proceeds progressively as the beer rises upward through a dense yeast plug. The yeast tends to settle back against the flow and very high cell densities (50-80 g l⁻¹) are achieved without the need for centrifuge cell recycle. Since volumetric productivity increases in proportion to active yeast cell density, very high productivities are achieved. The residence time for complete sugar utilization to produce a 6.5 wt% beer is 4h. Yeast strains used have been limited to slower fermenting varieties which have the necessary flocculence properties and will produce beer of the desired flavour characteristics. Fermentation temperature is regulated by cooling jackets at ordinary low brewing temperatures (16-23 °C) for best flavour and this also reduces the specific ethanol production rate.

A major drawback of the APV system is the long time required for start-up. Two to three weeks are required to build up the desired high cell density and achieve stable operation. Continuous run times of 12 months and greater have been achieved and compensate for the slow start-ups.

Process modifications would be necessary for large-scale industrial ethanol production. Larger diameter columns with higher throughput would be advantageous. Higher product concentration would also be desirable.

Alcohol Recovery

Alcohol product recovery is energy intensive, typically accounting for more than 50% of the total fermentative ethanol plant energy consumption. When heat from burning of raw material residues (such as bagasse) is not available, this constitutes a significant operating cost. Depending on recovery system design, recovery equipment cost generally makes up 6-12% of the plant total capital investment.

Industrial alcohol is produced in various grades. The majority is 190 proof (95 vol% or 92.4 wt% minimum) alcohol used for solvent, pharmaceutical, cosmetic and chemical applications. Technical grade alcohol (containing up to 5% volatile organic aldehyde, esters and sometimes methanol) is used for industrial solvents and some chemical syntheses. A high-purity 200 proof anhydrous alcohol product (99.85 wt%) is produced for specialized chemical applications. For fuel use in mixtures with gasoline (gasohol), a nearly anhydrous (99.2 wt%) alcohol, but with higher allowable levels of organic impurities, is used.

By-product effects

Refinement of industrial alcohol from fermenter beer is further complicated by the presence of fermentation by-products and nonmetabolized feed components which must be removed.

Yeast cells, nutrient salts, residual sugars, organic acids and glycerol are of low volatility and are recovered in the stripper stillage. These components do have a small effect on the ethanol/water equilibrium. More important, though, are physical effects, especially promotion of foaming on the stripper trays and fouling and corroding of trays and heat transfer equipment.

Acetaldehyde (and smaller amounts of other aldehydes and volatile esters) are produced at a rate typically of 1 l per 1000 l of ethanol for molasses or grain fermentation and at higher rates for the fermentation of sulfite waste liquor. In sulfite liquor fermentation, methanol is also present. Acetaldehyde and methanol are very volatile and can be distilled from 95 wt% alcohol product. With a boiling point of 21 °C, however, condensation of pure acetaldehyde to provide a reflux is impractical and, instead, a portion of the alcohol product must be recovered as a separate 'heads' technical grade to carry away the aldehyde. A

supplemental, aldehyde distillation at elevated pressure or with a refrigerated condenser can then be performed.

Fusel oils are a fermentation by-product produced at a rate of 1-5 l per 1000l of alcohol depending on the substrate. Fusel oil is composed of a mixture of amyl (pentyl) and propyl alcohol isomers. Because of its unusual equilibrium behaviour, fusel oil concentrates on the lower plates of a rectifying column. Dry fusel oils boil in the range of 128-137 °C and hence do not distill over-head with the more volatile alcohol product. In the presence of water, however, the volatility of fusel oils is greatly enhanced with only 2.4% amyl alcohol in water exerting the same partial pressure as 90.7% amyl alcohol. Fusel oils, thus, are forced into the vapor phase in the stripping section and cannot be recovered as a bottoms product. Fusel oil miscibility with ethanol and water decreases at reduced temperatures and fusel oils are therefore separated by cooling and decanting fusel oil laden liquid bled from the rectifier lower plates.

Three-column Barbet system

For higher quality alcohol, the three-column Barbet system was commonly employed (Fig. 1.4). In this system the stripped beer is first purged of most aldehydes in a purifying column which then feeds a relatively dilute alcohol stream to a stripper-rectifier. The alcohol product is taken as a side draw a few plates below the head of the rectifier. A small purge of additional aldehydes from the head

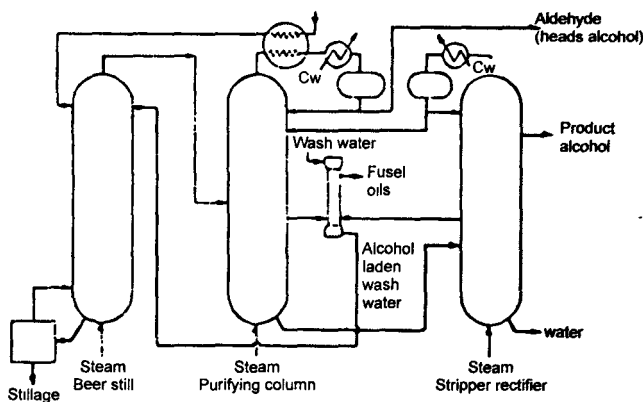


Fig. 1.4 : Three-column Barbet distillation system for 190 proof ethanol

Three-column Othmer system

In the 1930s an alternative three-column Othmer system incorporating vapour reuse methods to reduce steam consumption was developed (Fig. 1.5). A third aldehyde stripping column is added to the basic two-column design. The product alcohol side-draw from the rectifier is fed to the top plate of the aldehyde column and, in descending, is stripped of volatiles to produce an essentially pure azeotropic alcohol bottoms product. Aldehyde carrying vapours from the head of the aldehyde stripper are returned to the rectifying column and a technical grade heads alcohol product is tapped from the rectifier reflux to purge the aldehydes as in the simple two-column design. The main product is virtually free of aldehydes and is of higher quality than the Barbet system product.

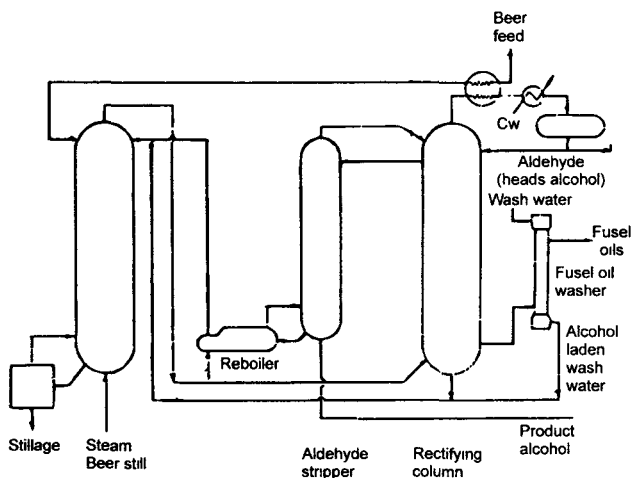


Fig. 1.5 : Three-column Othmer distillation system for 190 proof ethanol

Heat for alcohol vapour boil-up in the aldehyde stripper reboiler is provided by condensation of a portion of the stripper

vapour product, which is then returned to the stripper as reflux to reduce the stripper tray requirement. This reuse of heat increases energy efficiency and the steam requirement is not increased over the simple two-column design (2.4-3.0 kg per liter of 94 wt% product).

Vacuum rectification

Vacuum rectification can be used to reduce further the distillation energy requirement. The ethanol/water equilibrium is pressure sensitive, and the relative volatility of ethanol over water at high ethanol concentration is increased at low pressure. As the pressure is reduced from one atmosphere the azeotropic composition increases, the azeotrope disappearing at below 86 mmHg (11.5 kPa). Anhydrous alcohol can thus be produced by vacuum distillation, but the energy requirement for such a 'one-step' distillation is high and the process is uneconomical. Vacuum rectification to produce azeotropic alcohol is, however, very efficient with the required reflux ratio as set by the rectification head pinch reduced by 45% for distillation at 100 mmHg (13.3 kPa) compared with one atmosphere. A concentrated feed to the rectifier is necessary, though, to achieve these savings.

Anhydrous Ethanol Production

Azeotropic distillation

Azeotropic distillation is today the prevalent method for ethanol dehydration to produce anhydrous product. The benzene azeotropic distillation is exemplary (Fig. 1.6). Water is only slightly soluble in nonpolar benzene, while ethanol is freely soluble. The vapour pressure of water over a benzene/ethanol/water liquid mixture is thus greatly enhanced relative to the ethanol. In the distillation the ethanol/water mixture is fed at a midpoint in the column while an organic-rich phase (75 mol% benzene decanted from the cooled distillate) is used as reflux at the column head. Benzene concentrates in the liquid phase on the plates above the feed, enhancing the volatility of water and forcing it into the rising vapour phase. Ethanol concentrates with the benzene and is carried downward. The water-concentrated benzene/ethanol/water ternary azeotrope (24% ethanol, 54% benzene, 22% water) is taken as the column head product. Below the feed tray, benzene is

stripped from the ethanol and anhydrous ethanol is recovered from the azeotropic column reboiler. The distillate of the azeotropic column, when cooled and decanted, separates into two phases: the organic-rich phase used for reflux and a water-rich phase (35% ethanol, 4% benzene, 61% water) which is treated in the benzene recovery column. This column produces the benzene concentrated azeotrope as head product for recycle to the decanter and dilute alcohol as the bottom product. The dilute alcohol can be reconcentrated in an additional column or recycled to the primary alcohol recovery column. The distillation columns are costly as the azeotropic column alone requires 50 plates and is of similar diameter to the primary stripper rectifier.

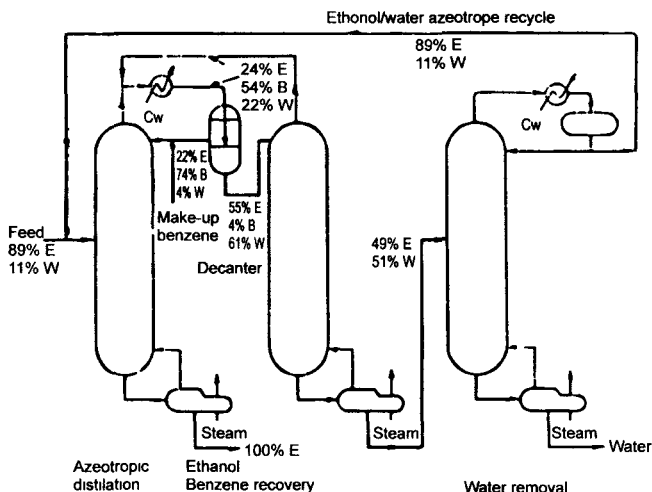


Fig. 1.6 : Benzene azeotropic distillation for anhydrous ethanol

In the US, anhydrous alcohol is generally refined from 94 wt% azeotropic alcohol. The additional steam requirement for anhydrous alcohol production with benzene as the entrainer is then 1.0 kg of steam per liter of product.

In Europe, a modification (the Mellé 5th method) has been widely used (Fig. 1.7). In this process a concentrating column, with the decanted benzene-rich phase as reflux, serves as a combined primary rectifier and benzene recovery column so that a dilute alcohol beer can be processed directly. The beer is fed to a stripper for removal of nonvolatile stillage. The stripper is also equipped with a small diameter rectifier for aldehyde heads impurities

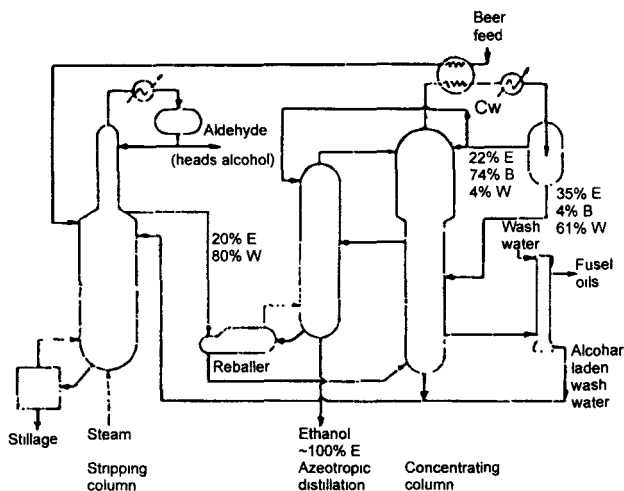


Fig. 1.7 : Mellé distillation system for anhydrous ethanol

removal. Dilute vapor (20% ethanol) from the top stripper plate is passed to the base of the concentrating column with a portion condensed in the azeotropic dehydration column reboiler for heat recovery. The concentrating column is a rectifier, and vapors become concentrated in alcohol as they rise. Fusel oils are tapped from a lower rectifying plate. The decanted water-rich phase is fed higher up in the rectifier so that benzene is also stripped in the lower section to produce a dilute, benzene free, liquid bottom product for recycle to the stripping column. Above the water-rich benzene phase addition plate the liquid phase becomes more and more concentrated in benzene and alcohol. A portion of this alcohol-rich liquid is tapped from the concentrator column and fed to the azeotropic dehydrating column, which produces anhydrous alcohol as the bottom product. Both the concentrating and dehydrating columns produce the ternary azeotrope as head products and these are condensed, separated into the organic and water rich phases, and recycled.

The steam requirement for this process (3.0 kg l^{-1} starting with a 6.5 wt% feed) is slightly lower than for separate production of 94 wt% ethanol and subsequent dehydration to anhydrous (3.4 kg l^{-1}). With efficient dual pressure vapor reuse methods, however, the steam can be largely reutilized if 94% and anhydrous alcohol production are carried out in a continuous process and the steam requirements of the two methods are then comparable.

Alternative Ethanol Recovery Methods

Extractive distillation using ethylene glycol, glycerine or a molten eutectic mixture of potassium and sodium acetate to depress the volatility of water and allow the distillation of anhydrous ethanol was widely practiced during World War II.

New nondistillative processes including dehydration by vapor phase water adsorption on to solids, molecular sieve drying, solvent extraction, extraction with supercritical fluids and membrane separations are all currently under study. Special processes for the production of gasohol using gasoline as an ethanol extractant are also under development.

Ethanol Recovery Process Comparison

Table 1.5 compares steam consumption and equipment requirements for several of the conventional distillation processes for ethanol recovery and purification. These processes are quite efficient, utilizing typically less than one-third the fuel value of the ethanol product in producing anhydrous ethanol from relatively dilute (6 wt%) fermentation beers. These processes also provide for the separate recovery of aldehyde, fusel oil and stillage by-products.

Table 1.5a : Conventional Ethanol Recovery Process Comparison : Azeotropic Ethanol Production

<i>Process</i>	<i>Steam consumption (kg t⁻¹ product)</i>	<i>Equipment</i>
Simple one or two column distillation to 94 wt% crude ethanol from 6 wt% beer	2.4-3.0	20 tray beer still 30 tray beer rectifier
Four column Barbet system for high quality 94 wt% neutral spirits from 6 wt% beer	4.0-4.2	20 tray beer still 30 tray purifying column 54 tray stripper rectifier small fusel oil column
Three column Othmer system for high quality 94 wt% neutral spirits from 6 wt% beer	2.4-3.0	20 tray beer still 45 tray aldehyde column 30 tray rectifier
Three column vacuum distillation for high quality 94 wt% neutral spirits from 6 wt% beer	1.8-2.2	20 tray beer still 45 tray aldehyde column 54 tray stripper/rectifier
Dual stripper, multieffect distillation for high quality 94 wt% neutral spirits from 1 wt%	8.3 (vs. 13.8 for a single stripper effect and 5.5 for three stripper effects)	Two 35 tray beer stills 45 tray aldehyde column 30 tray rectifier

Table 1.5b : Conventional Ethanol Recovery Process Comparison : Anhydrous Ethanol Production

<i>Process</i>	<i>Steam consumption (kg t⁻¹ product)</i>	<i>Equipment</i>
Benzene dehydration from 94-99.9 wt% ethanol	1.0	50 tray dehydrating column 30 tray benzene recovery column Supplementary rectifier
Pentane dehydration from 94-99.9 wt% ethanol	0.5	23 tray dehydrating column 18 tray pentane recovery column Supple- mentary rectifier
Ether dehydration from 94-99.9 wt% ethanol	0.6	60 tray dehydrating column 20 tray ether recovery column
Ethylene glycol extractive distillation from 94-99.9 wt%	1.1	50 tray dehydrating column 10 tray glycol recovery column
Mellé benzene process for 99.9 wt% ethanol from 6 wt% beer	3.1	Ethanol stripper/rectifier Concentrating column Dehydrating column
Combined Othmer three column and high-pressure ether distillation with vapor reuse for 99.9 wt% ethanol from 6 wt% beer	2.5	20 tray beer still 45 tray aldehyde column 54 tray stripper/rectifier 30 tray dehydrator 20 tray ether recovery column
Mariller glycerol extractive distillation process for 99.9 wt% ethanol from 6 wt% beer	3.4	Ethanol stripper/rectifier Extractive distillation column Glycerol recovery

Fermentation Waste Treatment and By-product Recovery

Introduction

Waste treatment and by-product recovery are extremely important factors in the economics of ethanol production. Secondary products recoverable from the feed during sugar preparation and prior to fermentation were considered under the feedstock alternatives sections. Here, postfermentation wastes and by-products are reviewed. The ethanol-depleted spent beer stillage is the most important waste stream. Without backset, typically 10-15l of stillage are produced for every liter of alcohol product. Corn stillage has a waste biological oxygen demand of 15 000-25 000 p.p.m. and a single 100 Ml y⁻¹ plant thus generates a pollution load

equivalent to a city of 1.4 million inhabitants. Balancing this, stillage can be treated to become a valuable by-product.

Fusel oil, aldehydes and carbon dioxide can also be recovered as valuable by-products.

Stillage Treatment and By-products

Stillage comparison

This section follows closely the recent review by Maiorella *et al.* (1983c).

Table 1.6 compares typical properties of stillage from fermentation of blackstrap molasses, corn and sulfite waste liquor. The biological oxygen demand (pollution load) varies from 60 000 for blackstrap molasses to 15 000 for corn stillage.

Table 1.6 : Properties of Ethanol Fermentation Stillages

	<i>Blackstrap Molasses</i>	<i>Sulfite waste liquor</i>	<i>Corn</i>
Biological oxygen demand (p.p.m)	50-60 000	40-50 000	15-25 000
Solids (wt%)	8.5	10	7.5
Ash (wt%)	2.5	2.1	1.5
Sugar (wt%)	0.9	0.8	0.5
Protein (wt%)	0.8	0.1	2.3
Vitamins	High	Low	High

Nutrient (protein and vitamin) content is highest for corn stillage containing corn protein residue from the germ and bran which are largely unaffected by the fermentation. Blackstrap molasses tillage is second highest in nutrient value with less protein than corn stillage, but high vitamin content. Mineral (ash) content is also high. Cane and beet juice stillages are similar in nutrient make-up to blackstrap stillage, but with lower nutrient concentrations. Potato stillage and cornstarch stillage (from distilleries with complete corn germ and bran removal prior to fermentation) are low in nutrient value. Sulfite liquor and wood hydrolyzate stillage are low in protein and vitamin nutrients but high in BOD, primarily as pentose sugars.

Stillage drying for cattle feed production

Fig. 1.8 shows the process for cattle feed production from corn stillage. Bulk solids are removed by centrifugation or by screening followed by dewatering in a rotary press. These solids then are dried to less than 5% water in rotary driers to produce the distiller's light grains product. The pressed liquid thin stillage is concentrated to 35% solids in forced convection vertical tube evaporators and then dried to a powder using drum or spray driers, yielding distiller's solubles. Alternatively, the concentrated liquid solubles can be blended back with the pressed solids and these rotary dried together to produce distiller's dark grains. About 700-900 g of stillage feed can be recovered per liter of alcohol produced.

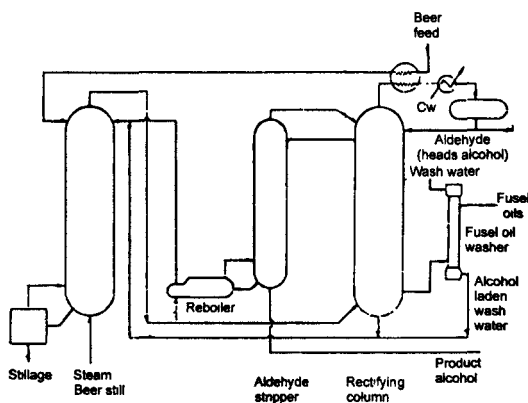


Fig. 1.8 : Three-column Othmer distillation system for 190 proof ethanol.

The feed value of corn stillage is similar to that of soybean (Table 1.7) and corn stillage is valued at approximately 95% of soybean cost for use in cattle feed.

The corn stillage cattle feed process recovers all the stillage solids, leaving no waste stream for further processing. The evaporation energy load to achieve this is, however, very high and a conventional plant may use as much steam for stillage drying as for alcohol distillation. To reduce steam consumption, vapor recompression heating of the evaporator can be used (Renshaw *et al.*, 1982). Steam consumption for the final grains drying has also been reduced by using carefully filtered hot boiler stack gases for rotary drier heating (Katzen, 1979). The total steam consumption for

cattle feed drying has thus been reduced to only 11% of the distillation steam consumption. An additional 111 kWh of electric power per metric ton of dried grains is consumed, primarily to drive the centrifuges and evaporator thick syrup recirculation pumps. This plant recycles 10% of the thin stillage as backset.

Table 1.7 : Nutrient Value of Grain Distiller's By-product

	<i>Distiller's solubles</i>	<i>Dark grains</i>
Moisture (%)	5	5
Protein (%)	27	29
Fat (%)	7	9
Fiber (%)	2	7
Ash (%)	8	4
Carbohydrate (%)	51	46
Vitamins (p.p.m.)		
Thiamine (B ₁)	8	4
Riboflavin (B ₂)	22	8
Pantothenic acid	29	11
Niacin	125	65
Pyridoxine (B ₆)	9	—
Biotin	0.5	0.2
Choline	6500	4500
Carotene	0.8	1
p-Aminobenzoic acid	10	—
Folic acid	4	—
Zeaxanthin	8	8
Cryptoxanthin	4	5
Price (\$ t ⁻¹) (1982)	185	191

Cane and beet juice stillages may be treated similarly to molasses stillage. Alternatively these stillages may be mixed with bagasse fiber or beet pulp residue and dried to produce a nutrient enriched roughage feed.

The soluble nutrient content of corn starch, potato and sulfite waste liquor stillage is not sufficient to justify the high cost of evaporation. For these stillages the yeast may be recovered by centrifugation and then spray or drum dried. Secondary treatment is required to reduce the stillage pollution load.

Secondary (aerobic) yeast production

Secondary aerobic yeast production is an effective means to reduce BOD and produce a valuable by-product. Sulfite liquor stillage and wood hydrolysis stillage are high in nonfermentable sugars from hemicellulose breakdown. Molasses stillage also has sufficient sugars, largely derived from hydrolysis of nonfermentables during distillation to make secondary fermentation economically attractive.

Stillage has also been used as fermentation medium for other important products. Grain stillage will support mold growth for amylase enzyme production. Supplemented grain and molasses stillage has been used for growth of *Penicillium notatum*. *Ashbya gossypii* growth to increase B vitamin content of stillage for cattle feed has also been practiced.

Anaerobic digestion

For stillage with low feed value but high BOD (*i.e.* corn starch, potato, sulfite waste liquor), anaerobic digestion can be cost effective. Low-grade distillery waste heat can be used to maintain high temperatures for rapid thermophilic (50 °C) digestion. Distillery waste is fed to large slowly agitated vessels and digested by a mixed culture sludge blanket the initial inoculum usually taken from a municipal sewage treatment facility. Acid forming bacteria convert sugar to acetic and butyric acids. Amino acids are broken down, releasing ammonium and sulfide ions. Methanogenic bacteria then convert the organic acids to methane and carbon dioxide; 580-720 l of gas, 65% methane, are produced per kg of BOD consumed. In an industrial, heated, agitated two-stage system with sludge recycle, yeast waste with a BOD of up to 15000 p.p.m. is treated in a two-day residence time with 90% BOD reduction. High BOD molasses distillery wastes are treated in 7-10 days. New reactor designs, such as the upflow reactor, are being evaluated at pilot plant scale and may greatly reduce the treatment time.

In some plants, ammonia recovery is also practiced (Skogman, 1979). The effluent liquid pH is increased to release ammonium ions as free ammonia gas. The liquid is stripped with air which is then counter current contacted with acid solution in an absorber to produce concentrated ammonium salts for recycle as a fermentation nutrient or for sale as a fertilizer; 90% nitrogen reduction in the stillage is typical.

For final BOD reduction an aerobic polishing stage is required. An aerobic activated biosludge reactor or a trickling bed reactor can be used. Loadings of up to $4.8 \text{ kg BOD m}^{-3}$ of stones in a trickling bed bioreactor can be handled. An overall BOD reduction for the complete system of 98-99% can be maintained. In the commercial ANOX process this effluent is further treated with ozone over a fixed bed metal catalyst for essentially complete waste oxidation. The resulting water is of drinking quality.

Typical biogas production rates for molasses, sulfite waste liquor and corn starch stillages, respectively, are 290, 230 and 80 l gas l^{-1} alcohol produced. This gas (65% methane), with a net heating value of approximately $25\,000 \text{ kJ m}^{-3}$, can be burned for steam or electric generation. For biogas from digestion of molasses stillage wastes with an initial BOD of $50\,000 \text{ p.p.m.}$, 2.9 kg of steam can be generated per liter of alcohol.

Stillage disposal as agricultural irrigant and fertilizer

Molasses stillage is high in minerals with as much as 1.1% potassium and 3.1% total ash. Repeated growing cycles deplete the soil of necessary minerals. The production of 100 tons of cane, for instance, is accompanied by a loss of 250 kg of K_2O . Direct disposal of untreated molasses stillage back to the crop lands is widely practiced.

After removal of valuable yeast, the stillage is trucked to cane plantations. In Brazil $650\text{--}1000 \text{ m}^3$ of raw stillage is spread per hectare, preferably 1-4 weeks prior to cane planting. In France, much lower levels ($2.5\text{--}30 \text{ m}^3 \text{ ha}^{-1}$) are sprayed on the fields after harvesting. Advantages cited (Chen, 1980) are: (1) disposal of the effluent; (2) replenishment of depleted minerals; (3) initial increase in soil pH; (4) improved soil physical properties; (5) increased water and salts retention capacity; (6) increased soil microflora population. These positive factors have combined to increase crop yield.

Strong odor and insect problems are associated with spreading of untreated stillage. Also, our experience has shown that while an advantage may be gained initially, stillage use must be severally reduced after several years due to problems of soil acidity increase, salt leaching and putrescency. The high concentration of calcium and magnesium in stillage initially acts to buffer the soil and beneficially increase pH. Repeated applications, however, lead to a

build-up of sulfates. These are reduced in the soil to hydrogen sulfide (giving a pungent odor), which is then reoxidized by sulfur bacteria to sulfuric acid. The problem of sulfite build-up rules out untreated sulfite waste liquor or wood acid hydrolysis stillages from direct applications to crop lands.

Stillage incineration

Stillage incineration can be attractive as a means to recover the mineral content of stillage with total consumption of the organic content. For stillages of high organic content (such as molasses, waste sulfite liquor and wood acid hydrolysis wastes) a positive energy return is possible. This process has been used commercially.

Stillage is concentrated typically to 60 wt% solids before incineration. The heat of combustion of molasses or cane juice stillage solids is 12 500-15 000 kJ kg⁻¹ and combustion generates sufficient heat for the evaporation step when a four or five-effect evaporator is used. The heat generated by sulfite waste stillage combustion is even greater.

Ash recovery is important to the economics of incineration processes. Molasses or cane juice ash is typically 30-40% potash (K₂O) and 2-3% P₂O₅. After dissolving in water and neutralization with sulfuric acid, a high-value potassium fertilizer is produced with 25-35 kg recovered per 1000 m³ of stillage incinerated. The potassium sulfate salt is contaminated with typically 16% potassium chloride and 7% potassium carbonate, reducing the sale value from \$212 per metric ton for refined potassium sulfate to \$120 per metric ton for the mixed salts. Additional ammonium sulfate can be recovered by absorption of sulfur dioxide from the boiler gas. Sodium sulfite ash is recovered from waste sulfite stillage incineration and recycled for use in the paper manufacturing process.

Distillation By-products

Recovery of acetaldehyde and fusel oils in the distillation of ethanol was described earlier.

Typically only 1 l of acetaldehyde and 5 l of fusel oil are produced per 1000 l of alcohol. Glycerol is produced at a rate of 41 l per 1000 l of alcohol but its recovery from the stillage is generally not economical.

Carbon Dioxide

Carbon dioxide is produced at a rate of 770 g l⁻¹ ethanol during fermentation. The carbon dioxide vapor is saturated with water and carries traces of ethanol, acetaldehyde, organic acids, fusel oils and compressor lubricating oils (CO₂ from sulfite liquor fermentation also contains methanol and hydrogen sulfide). After countercurrent absorption with water for alcohol recovery, further impurities are removed by absorption over activated carbon (Backus process) or by oxidation with potassium dichromate solution followed by sulfuric acid scrubbing for dehydration and dichromate removal (Reich process) 80% of the total fermentation CO₂ produced can be recovered at very high purity, and purification costs are lower than for CO₂ recovered from flue gases.

Electricity

Cogeneration can greatly improve the usable energy return in alcohol production. Alcohol production typically requires 3.0-5.0 kg steam per liter of alcohol depending on the process.

In a cogeneration scheme, fuel is burnt to produce high-pressure superheated steam which drives turbines for electric power generation. The low-pressure turbine exhaust steam is then reused to provide the process heat for distillery operation. Using a 40 atmosphere boiler, 0.17 kWh of electricity can be generated for every kg of process steam. A 100 Ml y⁻¹ alcohol plant boiler can thus also power an 8 MW electric generator. The favorable economic return for cogeneration is well proven, and in Hawaii 16% of all power is produced by cogeneration in sugar mills using bagasse fuel.

Ethanol solvent use

Ethanol ranks second only to water as an industrial solvent. Solvent applications include resins, pharmaceuticals, cosmetics, household cleaning products and industrial solvents; 50-55% of industrial (nonfuel) alcohol use is for solvent applications, as summarized in Table 1.8. Solvent usage is mature with few major new applications emerging and a slow growth rate of 2-3% per year is expected.

Table 1.8 : United States Ethanol Solvent Consumption (1975)

Use	Volume (MI)
Resins and lacquers	12.9
Pharmaceuticals and cosmetics	142.7
Cleaning preparations	80.2
Industrial solvent	47.3
Proprietary solvent formulation	91.2
Total	385.7

Ethanol use as chemical intermediate

Figure 1.9 summarizes several of the more important chemicals that can be derived from ethanol.

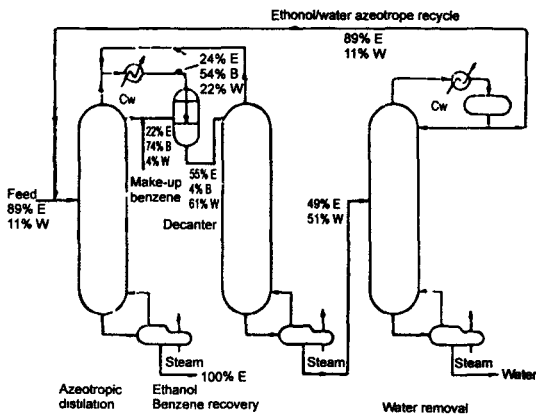


Fig. 1.9 : Benzene azeotropic distillation for anhydrous ethanol.

Ethanol fuel use

Fuel use in internal combustion engines is the fastest growing application for fermentative ethanol. The original Otto engine was developed using anhydrous ethanol fuel. Ethanol use in blends of up to 20 vol% with gasoline was widespread during World War II induced petroleum shortages. As early as 1931, Brazil legislated the use of a 5 vol% alcohol blend with gasoline as a means to utilize sugar refinery molasses waste and to stabilize sugar prices.

2

Wine and Brandy

The use of wine goes back to times immemorial; the bible, Homer's epics, Egyptian and Assyrian documents mention it. However, one must wait for the time of the Middle Ages before the alchemists discover the active principle, ethanol.

Brandy is obtained by distillation of wines. Throughout the Middle Ages the use of brandy remains strictly medicinal, and its production remains quasi secret. It changes from the art of the alchemist to an industrial art thanks to the work of the physician BOERHAVE during the 18th century. The "brandwine" (burnt wine) diluted with water became during the 17th century the normal beverage of Dutch sailors. The tonic can be stored in concentrated form without change, and it is indispensable in the fight against the epidemics of tropical countries. A lack of sales caused by wars leads to the discovery that brandy ameliorates during aging. Today there are several kinds of brandy distinguished by their origin, the quality of the base wine, and the nature of the distillation. The best known are undoubtedly cognac and armagnac.

All prestigious products have been created empirically. They are "the fruit of patient care...the reputation of the great wine regions having advanced, and greatly thanks to chemical and biological studies". Therefore, one easily attributes the merits of wines to the quality of nature, and sometimes one questions the role of enology.

RIBEREAU-GAYON and PEYNAUD supply a precise answer to this question of their "Traite d' Œnologie" (1960), "A good wine or a great wine may be obtained without the aid of modern enology. However, the aid of modern enology is in general required to obtain a better quality regularly and with certainty and with the most efficient means". And, finally, "enology is more than a specialty...it is a central interest around which one can create and coordinate a

comprehensive program of fundamental research which is authentic and of general interest".

YEASTS AND THE ALCOHOLIC FERMENTATION

Yeasts

Taxonomy, ecology

Soil yeasts are spread by wind and insects. Fruit flies and bees play a predominant role in their dissemination. Yeasts are present of vines from the start of ripening. The populations reach a maximum at the time of maturity. Yeasts are not numerous on stems and leaves where they form a pseudomycelium. Yeast cells can be recognized by their shape, the absence of ornamentation of their surface, and by their bud scars.

**Table 2.1 : Frequency of the Occurrence of Yeast Species
(expressed as a percentage of the number of samples tested)**

<i>Microorganisms</i>	<i>Domereq Bordelais 1956</i>	<i>Brechot Beaujolais 1962</i>	<i>Minarik Laho Czecho- slovakia 1970</i>	<i>Park Charentes 1974</i>
1	2	3	4	5
<i>Brettanomyces vini</i> or <i>B. intermedius</i>	0.2	2.5		
<i>Candida guilliermondii</i>		0.9		
<i>Candida intermedia</i>				0.3
<i>Candida krusei</i>		0.5	0.02	
<i>Candida melinii</i>		2.7		
<i>Candida mycoderma</i>			0.08	
<i>Candida parapsilosis</i>			0.02	
<i>Candida pelliculosa</i>		0.5		
<i>Candida sake</i>		1.5		0.6
<i>Candida solani</i>		0.5		
<i>Candida valida</i>				4.6
<i>Candida vini</i>		9.2		
<i>Candida utilis</i>		0.5		
<i>Debaryomyces hansenii</i>	0.2			0.15
<i>Debaryomyces phaffii</i>				
<i>Hansenula anomala</i>	0.3	0.2	1	0.15
<i>Hanseniaspora osmophila</i>				2.1

contd...

Table 2.1 – contd...

1	2	3	4	5
<i>Hanseniaspora uvarum</i>	27.8			
<i>Kloeckera apiculata</i>		6.5	13	15.5
<i>Kloeckera africana</i>	0.04		0.02	
<i>Kloeckera javanica</i>	0.04	0.5		
<i>Rhodotorula glutinis</i>				0.15
<i>Rhodotorula mucilaginosa</i>		0.2		
<i>Kluyveromyces veronae</i>	0.04		0.08	
<i>Metschnikowia pulcherrima</i>	0.29		9	0.3
<i>Nadsonia elongata</i>				0.15
<i>Pichia fermentans</i>	0.19		0.08	
<i>Pichia membranaefaciens</i>	0.19		0.2	
0.4				
<i>Pichia polymorpha</i>				0.15
<i>Pichia kluyveri</i>				0.4
<i>Saccharomyces bailii</i>	0.4		0.1	
<i>Saccharomyces bisporus</i>		0.2		
<i>Saccharomyces chevalieri</i>	3.5	1.5	0.2	1.4
<i>Saccharomyces capensis</i>				0.9
<i>Saccharomyces cerevisiae</i>	50	58	64	50.0
<i>Saccharomyces cidri</i>				0.4
<i>Saccharomyces delbrueckii</i>	0.04			
<i>Saccharomyces diastaticus</i>				0.3
<i>Saccharomyces exiguus</i>		0.2	0.2	
<i>Saccharomyces florentinus</i>	0.29			
<i>Saccharomyces globosus</i>				0.6
<i>Saccharomyces heterogenicus</i>	0.2	0.2	0.08	
<i>Saccharomyces italicus</i>	0.9	7.5	0.1	
<i>Saccharomyces kluyveri</i>				1.5
<i>Saccharomyces oviformis</i>				
and <i>S. bayanus</i>	4.8	0.5	4.5	0.6
<i>Saccharomyces pretoriensis</i>				0.4
<i>Saccharomyces prostoserdovii</i>				0.15
<i>Saccharomyces rosei</i>	1.6	0.2	0.9	7.3
<i>Saccharomyces uvarum</i>	0.9		3	6.2
<i>Saccharomycodes ludwigii</i>	0.04			1.8
<i>Torulopsis candida</i>	0.2	0.5		0.3
<i>Torulopsis colliculosa</i>		0.9		

contd...

Table 2.1 – contd...

1	2	3	4	5
<i>Torulopsis dattila</i>		0.2		
<i>Torulopsis etchellsii</i>				0.4
<i>Torulopsis glabrata</i>			0.08	
<i>Torulopsis holmii</i>		0.2		
<i>Torulopsis inconspicua</i>				0.15
<i>Torulopsis lactis condensii</i>				
<i>Torulopsis stellata</i>			0.08	2.1
<i>Torulopsis versatilis</i>	6.9	1.7	0.2	
Number of isolated strains	2023	398	3739	650

Table 2.2 : Media and Culture Conditions for the Selective Determination of Cell Counts for Yeasts, Lactic Acid Bacteria and Acetic Acid Bacteria

Micro-organism	Medium	pH	Antibiotic or Antiseptic	Active Against	Culture Condition
Yeasts	Grape Juice, 2 fold dilution	3	Diphenyl (12 mg/L)	Molds	Aerobic
Lactic acid bacteria	Synthetic Medium: yeast extract 0.5%; enzyme hydrolyzed casein 1.0%; glucose 1%	4.5	Pimaricin (50 mg/L)	Yeasts, molds	Under CO ₂ atmosphere
Acetic acid bacteria	Synthetic medium: yeast extract 0.5%; enzyme hydrolyzed casein 1.0%; glucose 1%	4.5	Penicillin	Lactic acid bacteria	Aerobic
			Pimaricin	Yeasts, molds	

Each medium is solidified by the addition of an equal volume of 2% agar solution.

On grapes one finds essentially molds, the yeast *Aureobasidium pullulans*, and yeast species with oxydative metabolism (*Rhodotorula*) or with little fermentation activity. Among the latter, *Hanseniaspora apiculata* predominate (99% of the isolated yeasts). *Metschnikowia pulcherrima*, *Pichia membranaefaciens*, and *Hansenula anomala* are less frequent; and other species may occur fortuitously.

All studies confirm the extreme rarity of the occurrence of *Saccharomyces cerevisiae* on grapes. The essential yeast microflora on other supports (beams, vaults, cellar walls consists of *Hanseniaspora*



Fig. 2.1 : *Saccharomyces cerevisiae*

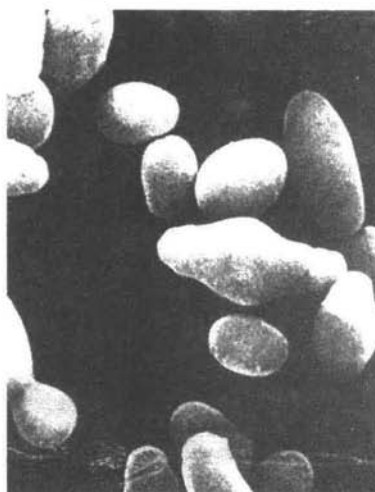


Fig. 2.2 : *Saccharomyces ludwigii*

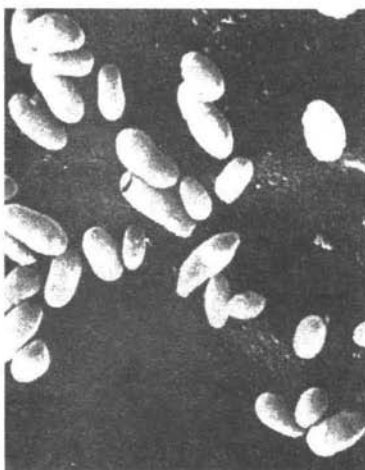


Fig. 2.3 : *Brettanomyces*



Fig. 2.4 : *Zygosaccharomyces*

Table 2.3 : Differentiating Characteristics of the Principal wine yeasts

	Morphology	Fermentation of Sugars						Assimilation of Sugars						
		Gl	Ga	Ma	S	L	R	T	Ga	Ma	S	L	R	T
<i>Saccharomyces cerevisiae</i>	ovoid	+	+	+	+	-	1/2	±	+	+	+	-	+	±
<i>Saccharomyces bayanus</i>	ovoid	+	-	+	+	-	1/3	±	-	+	+	-	+	±
<i>Saccharomyces rosei</i>	ovoid	+	-	-	+	-	1/3	+	-	±	+	-	+	+
<i>Saccharomyces uvarum</i>	ovoid	+	±	±	+	-	+	±	+	+	+	-	+	+
<i>Zygosaccharomyces</i>	ovoid	+	-	-	+	-	+	±	±	-	±	-	±	±
<i>Torulopsis stellata</i>	budding star shaped	+	-	-	+	-	+	-	-	-	+	-	+	-
<i>Hanseniaspora uvarum</i>	apiculate	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>Saccharomycodes ludwigii</i>	apiculate	+	-	-	+	-	+	+	-	-	+	-	+	-
<i>Brettanomyces vini</i>	formation of pseudo mycelium	+	+	+	+	+	-	+	+	+	+	+	+	-
<i>Hansenula anomala</i>	formation of pseudo mycelium	+	±	+	+	-	1/3		±	+	+	-	+	+
<i>Pichia membranaefaciens</i>	formation of pseudo mycelium	±	-	-	-	-			-	-	-	-	-	-
<i>Schizosaccharomyces</i>	cylindrical; division by fission	+	-	+	-	1/3	-	-	-	+	+	-	+	-

Gl = Glucose; Ga = Galactose; Ma = Maltose; S = Saccharose; L = Lactose; R = Raffinose; T = Trehalose

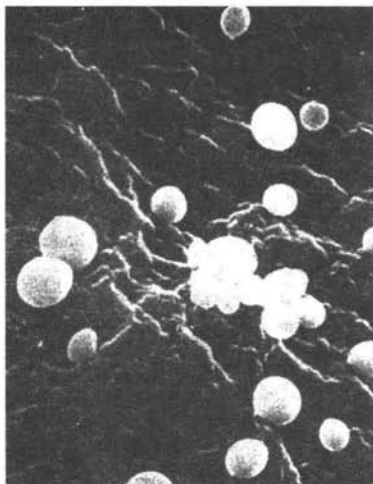


Fig. 2.5 : *Torulopsis stellata*

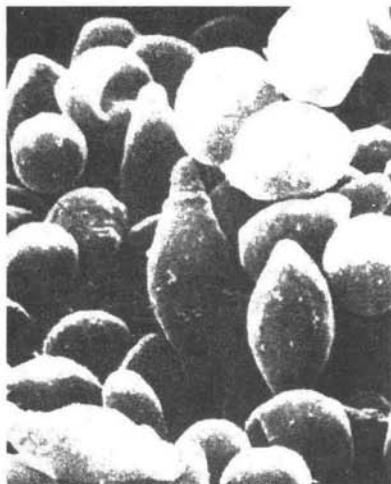


Fig. 2.6 : *Hanseniaspora uvarum*

uvarum, *Hansenula anomala*, *Metschnikowia pulcherrima*, *Pichia fermentans*, and *P. membranaefaciens*).

Industrially Important Yeasts

The principal yeasts fermenting must and responsible for biochemical changes in wines can be identified on the basis of a limited number of criteria (morphological and physiological) (Table 2.3). The effect of their occurrence on the essentials of wine quality is known.

Killer (K) yeasts

These yeasts are capable of excreting a toxin that is lethal for so-called "sensitive" (S) strains. The so-called "neutral (N)" strains do not produce the toxin and are resistant to it.



Fig. 2.7 : *Schizosaccharomyces pombe*

Killer yeasts can be found among the following genera; *Saccharomyces*, *Debaryomyces*, *Torulopsis*, *Candida*, and *Pichia*.

The K toxin is a glycoprotein which adheres to the cytoplasmic membrane. After a variable time of latency one finds an arrest of the synthesis of macro-molecules, an outpouring of compounds such as ATP and K^+ ions and an inhibition of the active transport system for amino acids.

Yeast Nutrients in Grape Musts

Composition of Grape Musts

From the point of view of the chemist, the must from grapes can be defined as an aqueous solution of sugars, organic acids and mineral salts. It also contains nitrogenous compounds, phenolic compounds, vitamins of the B complex and volatile substances. The pH lies between 2.8 and 3.9. The composition of musts varies depending on the grape variety, the nutrients available to the vines, the maturity and the sanitary condition of the grapes, and the means of extraction of the must from the grapes.

The sugars are represented by two hexoses, glucose and fructose, and two pentoses, arabinose and rhamnose. Glucose constitutes 85% of the sugars in unripe grapes. During ripening the concentration of fructose increases. At the time of maturity the ratio of the two sugars is close to 1 with a slight excess of fructose. In Californian musts a ratio of fructose/glucose varying from 0.83 to 1.12 has been determined. The concentration of reducing sugars (Table 2.4) varies from 120 to 250 g/L (Vogt *et al.*, 1974); that of pentoses is close to 1 g/L. In overripe grapes infected with *Botrytis cinerea* the concentration of sugars can reach 400 g/l.

Soluble polysaccharides (Table 2.5) are present in two forms (a) the pectins which are mainly built up from units of galacturonic acid partially esterified with methanol; (b) gums, that is, non-pectin polysaccharides, built up in chains containing galacturonic acid, pentoses (arabinose and rhamnose), and hexoses (galactose and mannose).

Table 2.4 : Composition of the Must of Red Varieties of the Bordelais Area (averages of several harvests)

	<i>Sugars</i> (g/L)	<i>Total Acidity</i> (meq/L)	<i>Tartaric Acid</i> (meq/L)	<i>Malic Acid</i> (meq/L)
Merlot	204	92	110	36
Cabernet Franc	192	99	101	40
Cabernet Sauvignon	194	102	111	50
Malbec	187	108	98	55
Petit Verdot	212	120	120	60

Table 2.5 : Polysaccharides of Grape Musts

	<i>Polysaccharides</i>			<i>Composition of Monomers of Polysaccharides (%) other than Glucans and Pectins</i>					
	<i>Glucans</i> (mg/L)	<i>Pectins</i> (mg/L)	<i>Other Polysaccharides</i> (mg/L)	<i>Ara</i>	<i>Rha</i>	<i>Ga</i>	<i>Man</i>	<i>Gl</i>	<i>Gal</i>
Sound grapes	0	670	340	24.1	4.0	52.1	2.3	3.8	13.8
Grapes infected by <i>Botrytis cinerea</i>	387	0	627	17.4	9.4	36.0	18.0	5.7	13.2

Ara = Arabinose; Rha = Rhamnose; Ga = Galactose; Man = Mannose; Gl = Glucose, Gal = Galacturonic acid

Tartaric and malic acids are the principal acids of must (Table 2.4). Citric acid is also present but in low concentration. The concentration of tartaric acid in the berry depends closely on climatic conditions. It is lowered in dry and sunny weather and higher after rains when the circulation of water in the vines increases. It varies from year to year from 2 to 5 g/L. Tartaric acid, a strong acid, plays a determining role in the structure of the wine. The concentration of malic acid depends also on climatic conditions but also on the grape variety and fertilization of the vine it varies from 2 to 7 g/L. The concentrations of citric acid are of the order of 200 to 300 mg/L but higher in musts infected with *Botrytis cinerea*.

Mineral salts are represented by phosphates, sulfates, chlorides, and silicates of potassium, and magnesium. Their concentration is higher in the ripest grapes.

It also depends on the relation between grape variety and soil.

**Table 2.6 : Evolution of B Vitamins During Ripening of Grapes
(averages of several harvests in several vineyards)**

	<i>Begin of Ripening</i>	<i>10 Days</i>	<i>20 Days</i>	<i>30 Days</i>	<i>40 Days</i>	<i>50 Days (Maturity)</i>
Thiamin (µg/L)	237	284	277	371	419	354
Riboflavin (µg/L)	7.0	8.0	9.3	7.5	7.0	6.6
Pantothenic acid (mg/L)	0.72	0.84	0.84	0.92	0.92	0.95
Nicotinamide (mg/L)	0.77	0.80	0.82	0.86	0.74	0.78
Pyridoxine (mg/L)	0.24	0.33	0.38	0.39	0.32	0.37
Biotin (µg/L)	5.0	4.0	2.3	2.8	2.7	3.2
Mesoinositol (mg/L)	286	311	351	380	398	388

The 10 vitamins of the B complex are present in must in concentrations varying, depending on the vitamin, from a few µg to a few mg/L (Table 2.6). The concentration of vitamins drops as grapes approach maturity. Over-ripe grapes show a loss of vitamins.

With regard to nitrogenous substances, only about a quarter of the nitrogen of the grape passes in solution into the must. The soluble nitrogen, from 100 mg to 1 g/L, is present in different forms. Free ammonia which is always present constitutes 3 to 10% of the total nitrogen; amino acids 25 to 30% (Table 2.7); proteins 5 to 10% and polypeptides 25 to 40%. The concentration of amino acids in berries increases until maturity is reached with an accumulation of up to 500 mg of proline per liter. In musts of grapes invaded by *Botrytis cinerea* the concentration of amino acids can be reduced by one half. The amino nitrogen is mainly accounted for by 4 amino acids which can reach a concentration of several hundred mg per liter; arginine, proline, threonine, and glutamic acid. The use of fertilizers and the variety of the grapes affect the concentration of amino acids in musts.

Aromatic substances have been mainly studied with aromatic grape varieties of the muscat type. These are terpenes (Table 2.8) of which 8 have been identified: linalool, geraniol, nerol, α-terpineol, and the furanic and pyranic oxides of linalool, which are mainly found in the skins. The loss of the aromatic character during seeding of the grapes and during fermentation is due to transformations of these terpenes.

Table 2.7 : Concentration of Free Amino Acids in Grape Musts (mg/L)

Amino Acid	Variety : Merlot				Variety : Cabernet Sauvignon				Average Concentration
	1	2	3	4	5	6	7	8	
Arginine	890	186	282	416	140	286	130	290	327
Aspartic acid	12	0	0	0	0	7	0	0	2
Glutamic acid	190	262	196	0	216	222	160	139	173
Cystine	0	0	0	0	0	0	0	0	0
Glycine	16	10	22	26	26	29	22	25	22
Histidine	20	5	9	11	7	15	10	15	11
Isoleucine	0	0	0	12	32	12	0	0	7
Leucine	28	19	14	19	9	32	18	20	20
Lysine	0	0	0	0	74	54	0	0	16
Methionine	4	0	3	3	0	0	0	0	1
Phenylalanine	18	1	0	0	0	15	0	7	5
Proline	500	440	190	0	420	216	364	0	266
Serine	80	80	65	64	62	64	65	74	69
Threonine	220	230	320	356	226	256	218	244	258
Tryptophan	0.4	0.2	0.6	2.1	1.4	0.3	0	0	0.6
Tyrosine	0	0	0	0	0	0	0	0	0
Valine	20	3	0	24	0	10	0	0	6

Table 2.8 : Terpene Compounds in Musts and Wines (maximum concentrations in µg/L for analyses carried out from 1970-1979)

Grape Variety	Linalool	Gera-niol	Nerol	α -Terpi-neol	Oxide of Lina-lool	Hotrienol
Muscats	1850	1850	450	1100	1140	+
Riesling	150	100	20	45	80	+
Gewürztraminer	135	150	40	20	t	+
Müller-Thurgau	30	65	t	8	t	+
Sylvaner	30	40	t	30	t	+
Muscadelle	50	25	t	35	t	t
Semillon	10	t	t	10	t	t
Chardonnay	t	t	t	35	0	t
Chenin	t	t	0	5	0	0
Cabernet Sauvignon	t	0	0	5	0	0
Dabouki	12	18	0	7	0	t
Ravat 6	25	30	0	12		t

t = traces (less than 5 µg/L); + = compound not determined

Terpene compounds could not be detected in the following varieties : Sauvignon, Ugni blanc, Cabernet Franc, Merlot rouge, Grenache, Chasselas, Dattier de Beyrouth.

Nutritional Requirements of Yeast and Their Provision in Musts

The yeasts, which are chemo-organotrophic microbes, derive their simple elements and their energy, which are required for the synthesis of their cell substance, from the degradation of complex organic substances. Sugars, the main source of carbon and energy, are present in the must as glucose and fructose. Pentoses are not fermented. Nitrogen which is indispensable for the synthesis of proteins is present as ammonia and as amino acids. Yeasts can synthesize all required amino acids from ammonia or ammonium salts. The principal starting point of this synthesis is glutamic acid which is formed from α -ketoglutaric acid. Supplementation of the must with phosphate is authorized by the Common Market. It must not exceed 30 g/hL. Amino acids can be directly assimilated or after deamination, decarboxylation, or transamination. Not all of the essential amino acids are always present in must. During the maturation of grapes the nitrogen, with the accumulation of proline, assumes a less readily assimilated form.

The functional role of sterols has now been elucidated. Ergosterol, the principal sterol of cellular membranes of the yeast, is formed from its precursor, lanosterol, and the latter is derived from squalene. The cyclization of squalene and the appearance of the function of a secondary alcohol can only take place in the presence of molecular oxygen.

Growth of Yeasts and Alcoholic Fermentation

Growth cycle of yeasts and kinetics of the fermentation

In order to study the development of indigenous yeast populations during the fermentation one can look at an extreme case of a must very rich in sugars. This was a must of 320 g sugar per liter, not well clarified, extracted from white grapes infected with *Botrytis cinerea* a few days after harvesting.

The inoculum is important, $2 \cdot 10^6$ cells/mL. These are yeasts of the grapes which inoculate the must. Yet from the first manipulations of the grapes the fermenting species, which are few on the grapes, multiply preferentially. The yeasts invade the crushed grapes and contaminate the must. Consequently, the initial populations vary throughout the wine making campaign both in

numbers and in their type. During holding of the grapes in the cellar they multiply by a factor of 10. Sulfiting and clarification of the must reduce their numbers. Nevertheless, after several days following harvest and in relatively mild weather one finds a population of the order of 10^6 cells per mL in the must (which is a rich inoculum) once it has been placed into the fermenter.

One distinguishes three principal phases of the growth cycle:

- (a) A phase of limited multiplication which lasts 2 to 5 days and which increases the population to the order of about 10^7 cells per mL.
- (b) A quasi stationary phase which lasts about 8 days.
- (c) A declining phase which progressively reduces the viable population to 10^5 cells per mL. This phase may last several weeks.

Fermentation Problems and Their Causes

Difficulties can arise at the start of the fermentation because of a relatively low temperature (less than 15°C), because of the presence of pesticide residues in the must, or equally because of an initial yeast concentration that is too small. The latter may occur in musts which have been clarified excessively or at the beginning of the fermentation season when fermenting microflora is insufficient. But the major microbiological problems arise actually when the fermentations "languish" and finally stop before all of the sugar has been fermented or before the desired equilibrium sugar / alcohol has been reached. From a technological point of view a number of factors can cause such difficulties during the fermentation. The following factors are the most important:

- a very high concentration of sugars in the must, either naturally or after addition of saccharose. At sugar concentration above 200 g/L the final stage of the fermentation is carried out by surviving populations with reduced fermentation activity. Above a certain sugar concentration the start of the fermentation is delayed, the speed of the fermentation is diminished and so is the total quantity of fermented sugar. At higher sugar concentrations the growth rate of yeasts is decreased.

Table 2.9 : Concentration of Endocellular Hexoses and Ethanol of *Saccharomyces cerevisiae* during a Grape Must Fermentation (results are expressed on the basis of 1 L of cellular water)

		Control Must	+ Ergosterol (50 mg/L)	Oleanolic Acid (50 mg/L)
1st Day	Hexises (g/L)	35	+	30
	Ethanol (g/L)	2	1.9	1.7
	Viable cells (10^6 /mL)	1.0	1.4	1.2
2nd Day	Hexoses (g/L)	22.5	30	35
	Ethanol (g/L)	3.9	6.0	6.6
	Viable cells (10^6 /mL)	3.5	3	2.9
3rd Day	Hexoses (g/L)	14.5	14.9	15.8
	Ethanol (g/L)	6.8	12.9	11.7
	Viable cells (10^6 /mL)	24	26	22
6th Day	Hexoses (g/L)	14	14.2	15.1
	Ethanol (g/L)	6.8	14.4	13.5
	Viable cells (10^6 /mL)	25	26	25
10th Day	Hexoses (g/L)	5.7	4.6	6.7
	Ethanol (g/L)	4	7.8	7.9
	Viable cells (10^6 /mL)	9.4	15.9	15.5
14th Day	Hexoses (g/L)	3.1	1.8	3.2
	Ethanol (g/L)	4.3	8.3	8.2
	Viable cells (10^6 /mL)	5	8.6	6
17th Day	Hexoses (g/L)	0	0	0
	Ethanol (g/L)	4.0	8.7	9.1
	Viable cells (10^6 /mL)	4.0	9	7
23rd Day	Hexoses (g/L)	0	0	0
	Ethanol (g/L)	4.5	8.5	9.5
	Viable cells (10^6 /mL)	1.2	6	4.4

Initial must sugar concentration : 260 g/L; industrial active, dry yeast *S. cerevisiae*; initial yeast population 5.6×10^5 cells/mL; temperature of the fermentation : 25°C; initial endocellular hexose concentration : zero.

• the ethanol produced by fermentation of sugars is a major inhibitor of yeast growth and fermentation activity. Its maximal accumulation in the cell precedes cessation of yeast growth. It causes inhibition of the alcohol dehydrogenase. The addition of

ethanol to a culture medium produces a lengthening of the period of latency, an increase in the generation time, and a decrease in the maximum population. The phenomenon is more pronounced the higher the concentration of ethanol. Higher alcohols also exert an inhibition which increases with the number of carbon atoms in the molecule and in inverse ratio to their water solubility.

The effect of tannins is controversial. The phenolic compounds derive their effect from the possibility of their combination with proteins. Certain phenolic compounds serve as yeast growth and survival factors for *Hanseniaspora uvarum*.

- anaerobic conditions due to very large volumes of must in large fermenters.

- a high fermentation temperature (above 35°C). The start of the fermentation and its rate are faster as the temperature increases from 15 to 30°C, cite the example of a must with 200 g/L of sugar whose fermentation required 4 days at 30°C, 2 weeks at 20 °C and several weeks at 10°C. The higher the sugar concentration in the must and the higher the temperature, the slower will be the fermentation and it may be arrested before the sugar has been completely fermented. The biomass was 3 g/L at 10°C, 2.6 g/L at 20°C and 2.1 g/L at 30°C. The authors relate this phenomenon to the accumulation of ethanol in the cells. Besides, the concentration of sterols of the cell decreases rapidly at higher temperatures. On the 4th day of the fermentation at 25°C, it was twice what it was at 35°C, under otherwise identical conditions (Table 2.10). The exhaustion of cellular sterols can also explain the premature arrest of yeast growth at temperatures above 35°C.

- an insufficient inoculum: (a) qualitatively; this is the case at the start of the vintage when yeasts with feeble fermentation activity predominate in the must; (b) quantitatively; after an excessive clarification of the must for white wine production.

- the presence in the must of inhibitory substances excreted by *Botrytis cinerea*. If one ferments different blends of musts from sound grapes and from infected grapes (by *B. cinerea*), at the same sugar concentration and at the same pH, one observes a slower and less complete fermentation as the proportion of infected must is increased. The inhibiting substances, similar to botryticin, turn out to be also acetogenic.

Table 2.10 : Influence of the Temperature on the Concentration of Sterols of yeasts and on Their Fermentation Activity.

<i>Temperature</i>		<i>3rd Day</i>		<i>6th Day</i>	
		<i>Control</i>	<i>+Cholesterol (50 mg/L)</i>	<i>Control</i>	<i>+Cholesterol (50 mg/L)</i>
25°C	Sugars fermented (g/L)	53	55	192	236
	Sterols (% of dry weight)	0.6	1.1	-	-
30°C	Sugars fermented (g/L)	87	90	169	192
	Sterols (% of dry weight)	0.5	1.1	-	-
35°C	Sugars fermented (g/L)	76	79	119	142
	Sterols (% of dry weight)	0.3	0.9	-	-

- the presence in the must of pesticide residues resulting from vineyard operations.

- the clarification of musts for white wine production. The mechanization of processes for the extraction of musts has occurred for the sake of simplification but it also generates "mud". These insoluble particles of plant origin cause deviations from the normal metabolism of yeasts and produce a heaviness and a characteristic taste of plant vegetation to the wine.

These qualities depend on the grape variety, on the maturity of the grapes, but largely on the mechanical treatment of grapes. Grapes which have been crushed vigorously yield a must high in sediment which, after clarification, has only moderate fermentability. The effect of the mechanical treatment of grapes on the fermentability of the must and the quality of the wine has only recently been observed. It must be considered in the choice of cellar equipment.

- the onset of the fermentation is much faster at higher pH values.

Stimulation of the Fermentation

In order to mitigate the difficulties of a fermentation the enologist must not only encourage cell multiplication by an activation of yeast growth—as has been known for a long time—but also and most importantly by retarding the inhibition of surviving populations until the wine has reached the desired concentrations

of ethanol and residual sugar. The means at one's disposal are the management of the yeast, the addition of chemical fermentation activators and aeration.

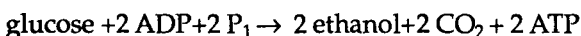
Yeast management. For maximum efficiency one must first stimulate the speed of the fermentation by an increase in the initial cell concentration. This is particularly important when the initial cell population is low (less than 10^4 cells/mL) for the first fermenters filled at the beginning of the season and mainly in cold weather.

Biochemistry of the Fermentation of Grape Must

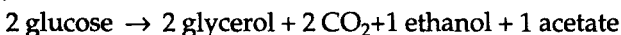
Primary and Secondary Products

The fermentation of glucose and fructose by yeasts can follow two pathways which are shown schematically by the following total equation;

(a) alcoholic fermentation



(b) glyceropyruvic fermentation according to Neuberg



The formation of acetate is attributed to a hydrolytic dismutation of acetaldehyde (reaction of Cannizzarro)



But this latter reaction could not be proven. It is a two-fold action of alcohol dehydrogenase and acetaldehyde dehydrogenase which reduce or oxidize, respectively, the acetaldehyde to ethanol.

In grape must about 8% of the fermentation of sugars follows pathway (b). The cause of the glyceropyruvic fermentation in grape must is still under discussion. Enologists generally consider it an induced process. During the first moments of glycolysis the acetaldehyde, which is the normal acceptor of electrons from glyceraldehyde phosphate, is not yet present. The dihydroxyacetone phosphate which has already been formed by splitting of the hexose diphosphate serves then as electron acceptor. It is reduced to glycerol by the alcohol dehydrogenase. Each time a molecule of glycerol is formed one molecule of pyruvic acid is liberated, and this serves as the basis for the formation of secondary products of the fermentation. It can be represented schematically as follows:

$$2a+5s+2m+b+h=g$$

where a acetic acid
s succinic acid
m acetoin
b 2,3-butanediol
h acetaldehyde
g glycerol.

The numerical coefficients represent the number of molecules of acetaldehyde (or pyruvic acid) required for the synthesis of each of the compounds.

Succinic acid is the principal acid formed during the fermentation of grape must (0.5 to 1.5 g/L). The succinic acid is derived from the oxidative degradation of glutamic acid and, to a lesser extent, via the reductive breakdown of aspartic and malic acids.

The carbonyl compounds, pyruvic and α -ketoglutaric acid as well as acetaldehyde, are always present in wine. Their excretion is sizable during the phase of yeast proliferation, but subsides towards the end of the fermentation. The liberation of acetaldehyde is increased by the presence of SO_2 in the must. That of the keto acids is increased by a higher pH, an elevated temperature, anaerobic conditions, and a lack of thiamin or pantothenic acid. Some yeasts, such as *Schizosaccharomyces* species, are particularly ketogenic. The addition of thiamin to the must prior to the fermentation prevents the accumulation of these compounds in the wine.

Acetic acid is the principal compound included in the analytically determined "volatile acidity". In wine it is essentially of bacterial origin. Its production by yeasts is generally slight and depends on the strain of yeast. Generally *Saccharomyces cerevisiae* forms only very small concentrations of acetic acid. But higher concentrations appear under anaerobic conditions, at pH values below 3 or above 3.4, at temperatures between 20 and 30°C, and in the absence of pantothenic acid. Also, the formation of acetic acid depends closely on the initial sugar concentration of the must (Table 2.11), but it is independent of the quantity of sugar actually fermented.

Table 2.11 : Effect of the Initial Sugar Concentration of a Must on the Formation of Secondary Products of the Fermentation (LAFON-LAFOURCADE, 1979)

<i>Sugar Initial</i> (g/L)	<i>Sugar Fermented</i> (g/L)	<i>Secondary Products Formed</i>		
		<i>Acetic Acid</i> (g/L)	<i>Glycerol</i> (g/L)	<i>Succinic Acid</i> (g/L)
224	211	0.26	4.79	0.264
268	226	0.45	5.33	0.251
318	211	0.62	5.70	0.256
324	179	0.84	5.95	0.262
348	152	1.12	7.09	0.283

Volatile Substances Contributing to the Aroma of Wine

Many compounds which are secondary fermentation products are among important contributors to the aromatic character of wines (secondary aromatic substances). Several hundred have been identified and the list keeps growing. They belong basically to three types of organic compounds, the esters, the higher alcohols, and the fatty acids (Tables 2.12, 2.13 and 2.14).

Table 2.12 : Concentration of Esters in Wine

<i>Ester</i>	<i>Concentration (mg/L)</i>			
	<i>White Wines</i>		<i>Red Wines</i>	
	<i>min.</i>	<i>max.</i>	<i>min</i>	<i>max.</i>
Methyl acetate	0	0.11	0.08	0.15
Ethyl acetate	4.50	180	22	190
Propyl acetate	0	0.04	0	0.08
2-Methyl-Propyl acetate	0.03	0.60	0.01	0.08
3-Methyl butyl acetate	0.04	18	0.04	50
Hexyl acetate	0	2	0	1.60
2-Ethyl acetate	0.20	5	0.10	5.0
Ethyl formate	0.20	0.84	0.03	0.20
Ethyl propionate	0	7.50	0.07	0.25
Ethyl 2-methy-propionate	0	0.60	0.03	0.08
Ethyl butyrate	0.04	1.0	0.01	0.20
Ethyl 2-methyl-butyrate	0	0.02	0	0.08
Ethyl 3-methyl-butyrate	0	0.04	0	0.09
Ethyl hexanoate	0.06	2.5	0.06	1.5
Ethyl octanoate	1.10	8.0	1.0	6.0
Ethyl decanoate	0.90	6.0	0.60	4.0
Ethyl dodecanoate	0.10	1.20	0.05	0.8
Ethyl tetradecanoate	0.10	1.20	0.05	1.0
Ethyl hexadecanoate	0.10	0.85	0.05	1.0
Ethyl lactate	3.80	50	9	50
Ethyl succinate	0.01	10	0.01	10.0

The ethyl esters of higher molecular weight are desirable elements of the aroma of wines to which they give a "flowery" or "fruity" odor. About 50 of these esters have been identified. In contrast ethyl acetate confers a disagreeable odor which is specifically vinegary.

Table 2.13 : Alcohols and Polyols of Wine

Alcohol	Concentration (mg/L)			
	White Wines		Red Wines	
	min.	max.	min.	max.
Methanol	20	118	43	222
Ethanol	9	48	11	52
2-Methyl 1-propanol	28	170	45	140
1-Butanol	0.5	8.5	0.5	2.3
2-Methyl 1-butanol	17	82	48	150
3-Methyl 1-butanol	70	320	117	490
1-Hexanol	1	10	12	10
2-Phenyl ethanol	15	250	42	129
Glycerol	5600	9460	7900	9200
2,3-butanediol (<i>levo</i> and <i>meso</i>)	300	600	486	570

Table 2.14 : Concentration of Volatile Fatty Acids in Wine

Acid		Concentration (mg/L)			
		White Wines		Red Wines	
		min.	max.	min.	max.
Acetic	C ₂	30.9	741.8	66.2	611.5
Propionic	C ₃	0.48	4.26	0.58	2.56
2-Methyl propionic	C ₄	0.22	6.83	1.32	5.93
Butyric	C ₄	0.25	4.62	0.19	3.29
3-Methyl butyric	C ₅	0.15	3.82	0.87	3.78
C ₃ +C ₄ +C ₄ +iC ₅		1.10	19.53	2.96	15.51
Hexanoic	C ₆	1.14	9.11	1.41	4.61
Octanoic	C ₈	1.29	13.95	1.42	6.22
Decanoic	C ₁₀	0.27	7.48	0.22	1.32
Dodecanoic	C ₁₂	0.10	0.96	0.06	0.27
C ₆ +C ₈ +C ₁₀ +C ₁₂		2.80	31.50	3.11	12.41

Lactic Acid Bacteria and the Malo-lactic Fermentation

Lactic Acid Bacteria of Wines

Taxonomy

The lactic acid bacteria of musts and wines have been extensively investigated. After isolating 750 cultures from wines of viticultural areas from all over the world these authors have proposed a system of classification which "can be well integrated with the general classification and yet conserves oenological relevance" (Table 2.15).

Table 2.15 : Classification of Lactic Acid Bacteria of Wine
(RIBERAU-GAYON *et al.*, 1979)

Cocci	homofermentative	<i>Pediococcus cerevisiae</i> <i>Pediococcus pentosaceus</i>
	heterofermentative	<i>Leuconostoc gracile</i> <i>Leuconostoc oenos</i> A+ <i>Leuconostoc oenos</i> X+ <i>Leuconostoc oenos</i> P+
Bacilli	homofermentative	<i>Lactobacillus plantarum</i> <i>Lactobacillus casei</i> <i>Streptobacterium</i> sp.
	heterofermentative	<i>Lactobacillus fructivorans</i> <i>Lactobacillus desidiosus</i> <i>Lactobacillus hilgardii</i> <i>Lactobacillus brevis</i>

The important documentation which has been assembled actually shows that only a restricted number of species develops in musts and wines since this medium is quite selective due to its acid pH and its alcohol concentration. These species belong principally to the genera *Pediococcus*, *Leuconostoc*, and *Lactobacillus* (Table 2.16).

Lactic acid bacteria are isolated on a solid nutritive medium of pH 4.5 in the presence of pimarin, an antibiotic and fungicide, and under light carbon dioxide pressure to inhibit acetic acid bacteria (Table 2.16). The identification is based on the following criteria:

- Morphology : the cells are spherical cocci of the genera *Leuconostoc* and *Pediococcus* or rods, the lactobacilli; nevertheless, (Figs. 2.8 and 2.9).

Table 2.16 : The Principal Lactic Acid Bacteria of Musts and Wines

	Form	Differentiating Pentoses Fermented Arabinose	Characteristics Xylose	Metabolic Pathway of Glucose Fermentation	Isomers of Lactic Acid Formed from Glucose	Frequency (%) of Total Species Identified (570)
<i>Pediococcus cerevisiae</i>	spherical	—	—	homo-fermentative	D(-); L(+)	4.8
<i>Leuconostoc gracile</i>	spherical	—	—	hetero-fermentative	D(-); 2% L(+)	20.6
<i>Leuconostoc oenos</i>	spherical	±	±	hetero-fermentative	D(-); 2% L(+)	37
<i>Lactobacillus casei</i>	rods			homo-fermentative	L(+); D(-)	2.1
<i>Lactobacillus fructivorans</i>	rods	—	—	homo-fermentative	L(+)	1.2
<i>Lactobacillus desidiosus</i>	rods	+	—	hetero-fermentative	D(-); 2% L(+)	3.6
<i>Lactobacillus hilgardii</i>	rods	—	—	hetero-fermentative	D(-); L(+)	0.4
<i>Lactobacillus hilgardii</i>	rods	—	+	hetero-fermentative	D(-); L(+)	22.5
<i>Lactobacillus brevis</i>	rods	+	+	hetero-fermentative	D(-); 2% L(+)	7.7

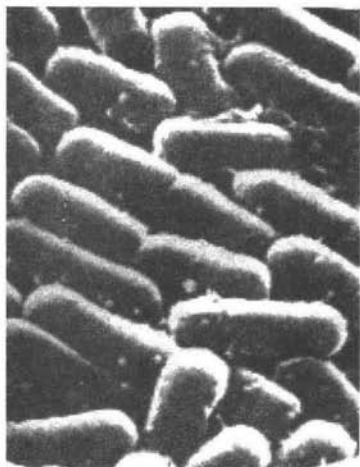


Fig. 2.8 : *Lactobacillus hilgardii*



Fig. 2.9 : *Leuconostoc oenos*

- The metabolic route for the assimilation of sugars. The only process is fermentative because a biosynthetic deficiency does not permit the synthesis of respiratory enzymes.

- The fermentation of pentoses. This leads to the formation of larger quantities of acetic acid. Wine contains from 0.26 to 1.65 g/L of arabinose and from 0-0.44 g/L of xylose.

Ecology

Lactic acid bacteria can be found on grapes but in lesser numbers than the acetic bacteria or the yeasts. They are also found on leaves.

The Role of Lactic Acid Bacteria in Vinification

In northern viticultural regions the malolactic fermentation of red wines is desired for two main reasons: (a) The bacterial transformation of malic acid to lactic acid deacidifies the wine conveniently and confers a mellowness to it; (b) the disappearance of malic acid provides a biological stability with regard to the action of lactic acid bacteria.

In white wines the malo-lactic fermentation occurs less frequently. Often one tries to produce "fresh" wines which can tolerate a certain acidity. However, the general direction is in the

development of wines with low acidity as demanded by consumers. The first experimental work done in Bordeaux shows that with a malo-lactic fermentation one can produce wines which are more mellow and with a more complex aromatic quality. However, under other conditions which have so far only been poorly defined (quality of strain, difficulty of growth) the malo-lactic fermentation can lead to volatile compounds which give the wine a "lactic odor" which is undesirable.

Traditionally, the malo-lactic fermentation is based on bacterial growth on malic acid. But in musts and wines the bacteria also find other substrates such as sugars, amino acids, organic acids, and glycerol. The metabolism of most of these compounds by lactic acid bacteria leads to spoilage. Particularly the degradation of sugars is a serious problem which leads to a "lactic flavor". One tries to prevent this by trying to delay the onset of the malo-lactic fermentation until all sugars have been fermented by yeasts, that is, to a level at which malic acid becomes the most readily fermentable substrate. But wine is not a particularly good growth medium. Consequently, the enologist is confronted with two problems which have not yet been solved well: (a) control of bacterial growth; (b) control of the nature of the degradable substrate.

In warm regions musts have only small concentrations of malic acid and a relatively higher pH. They are better suited for bacterial growth, but this would lead to an excessive deacidification of the wine and to spoilage. In some cases one tries to prevent the malo-lactic fermentation.

Bacterial Growth and Malo-lactic Fermentation

Development of Lactic Acid Bacteria During Vinification; Kinetics and Biochemistry of the Malo-lactic Fermentation

The following example concerns red grapes containing 200 g/l of sugar, and with a pH of 3.5; either sulfited at 10 g/hL or not sulfited, and fermented in vats under industrial conditions (Fig. 2.10). The indigenous population of lactic acid bacteria is initially of the order of 10^4 cells/mL in the control must. The number is reduced to 10^3 cells/mL by sulfiting. During the fermentation the numbers decrease to about 10^2 cells/mL

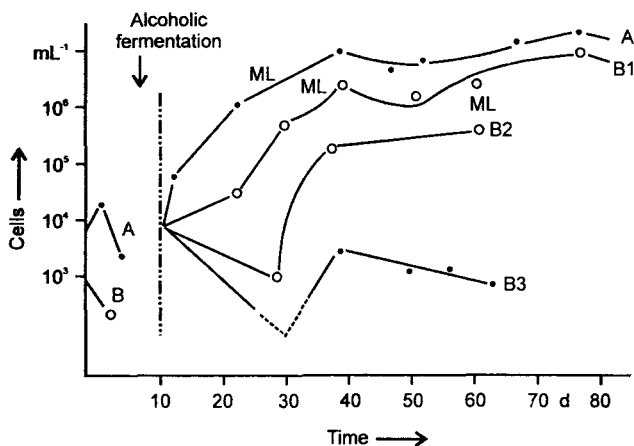


Fig. 2.10 : Development of populations of lactic acid bacteria during vinification.

A—●—grapes not sulfited, storage at 19°C and 14°C; B grapes sulfited (10 g/hL); B1—○—storage at 19°C; B2—□—storage at 14°C; B3—●—free run juice sulfited (5 g/hL), storage at 14°C; ML end of the malo-lactic fermentation.

The draining of the wine from the crushed grapes redistributes the populations. A part is eliminated with the pulp, but the wine is enriched with the microorganisms of the cellar. One finds again populations of 10^4 cells/mL. Four conditions can now prevail:

1. non-sulfited grapes, storage temperature 19 or 14°C. The growth of the bacteria becomes explosive and a population maximum of $3 \cdot 10^7$ cells/mL is reached in 2 weeks. The fermentation of malic acid is completed even before the stationary phase of bacterial growth is attained. The malolactic fermentation is completed about 12 days after draining of the wine.

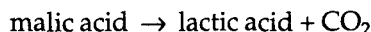
2. sulfited grapes, storage temperature 19°C. Growth of bacteria is slower, and maximal populations are up to 10^7 cells/mL. The degradation of malic acid is completed at the beginning of the stationary phase. The malo-lactic fermentation lasts an additional week.

3. sulfited grapes, storage temperature 14°C. One observes first a decline of the population to 10^3 cells/mL, and then a growth to no more than 10^6 cells/mL. The degradation of malic acid is prolonged during the stationary phase, and the malo-lactic fermentation is completed in 44 days.

4. sulfited grapes, the wine is sulfited with 5 g SO_2/hL after draining from the pulp, temperature of storage 14°C . The decline of the bacterial population is considerable. Population growth is limited to a maximum of 10^4 cells/mL. Two thirds of the original amount of malic acid remains in the wine after 44 days.

The kinetics of the malo-lactic fermentation is directly tied to the bacterial biomass formed, and the biochemical reactions are carried out by bacteria during their phase of growth. Hence, it is rapid. During the stationary phase or the declining phase it is much slower. Therefore, the occurrence of only a single unfavorable factor increases the generation time, limits total population growth and prolongs the malo-lactic fermentation.

The total equation for the malo-lactic fermentation has been known for a long time. In 1900 KOCH achieved the deacidification of wine at the expense of malic acid by inoculating with bacteria. In the following year SEIFERT gave the chemical equation:



Different metabolic pathways fit this equation (Fig. 2.11). Pathway (1) is based on the action of a malate dehydrogenase; pathway (2) on a malic enzyme. Both pathways require the formation of pyruvic acid as an intermediate compound. Lactic acid bacteria have L(+)- and D (-)-lactic acid dehydrogenases. The reduction of pyruvic acid can, therefore, lead to two forms of lactic acid isomers. Nevertheless, the bacteria of wine transform malic acid stoichiometrically to L(+)-lactic acid which suggests lack of lactic acid dehydrogenase activity, (3) in which lactate dehydrogenase does not participate. The enzyme is called "malo-lactic enzyme".

The malo-lactic enzyme has a molecular weight of 235000 and an iso-electric pH of 4.35. It may consist of a complex of enzymes to which the intermediary compounds, oxalacetate and pyruvate, remain bound. It could be constituted either by a malic enzyme and a lactate dehydrogenase, or by a malate dehydrogenase, an oxalacetate decarboxylase and a lactate dehydrogenase. The complex of enzymes is activated by NAD. Tartaric, succinic and lactic acids act as competitive inhibitors. It is constitutive in about 60% of the homo- and heterofermentative lactic acid bacteria.

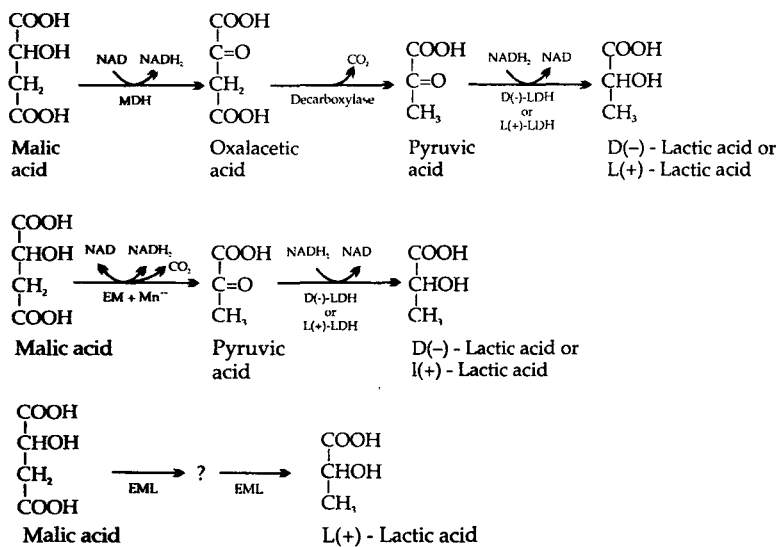


Fig. 2.11 : Metabolic pathways of the malo-lactic fermentation

Parameters Affecting the Development of Lactic Acid Bacteria in Wines

Such factors are nutritional and physicochemical. The energy requirements are relatively small. Others have calculated that 100 mg/L of carbohydrate are sufficient to supply energy for the growth of 100 mg/L of bacterial biomass (dry weight) which is capable of fermenting 1 g/L of malic acid. These requirements can be fulfilled by the residual sugars of the wine which consist of several 100 mg of fructose, glucose, arabinose, xylose, and trehalose. The malo-lactic fermentation is slightly exothermic; some have estimated the amount of energy liberated by the malolactic fermentation at 18.6°C, pH 3.4, and under a CO₂ atmosphere as 6 kcal (25.1 kJ) per molecule. It seems difficult to use this energy for growth. Nevertheless, this degradation of malic acid favours growth. According to Chauvet *et al.* (1980) malic acid can serve as a growth substrate for *L. oenos* under certain conditions.

The nitrogen source must be organic, either amino acids or peptides. Lactic acid bacteria cannot synthesize all of the required amino acids from other amino acids. Therefore, some amino acids are indispensable and can be considered growth factors. Glutamic

acid, iso-leucine, and valine have to be supplied to most lactic acid bacteria, as well as others depending on the strain. Requirements for cocci are still more stringent, and these require in addition arginine, histidine, methionine, phenylalanine, serine, tryptophan, and tyrosine. Purine and pyrimidine bases are often stimulants. The alcoholic fermentation rearranges and completes the amino acid composition of the must (Table 2.17).

The presence of lactic, succinic, and tartaric acids has an unfavorable effect on the malo-lactic fermentation. Tartaric acid under conditions of identical pH values reduces the amount of biomass formed (Table 2.18), and so does malic acid at high concentrations.

Table 2.17 : Concentration of Free Amino Acids in Red Wines of the Bordeaux Regino (concentration in mg/L)

<i>Amino Acid</i>	<i>Cotes de Canon-Fronsae</i>	<i>Saint-Emilion 1</i>	<i>Saint-Emilion 2</i>	<i>Saint-Emilion 3</i>	<i>Medoc</i>	<i>Average Concentration</i>
Arginine	25	66	49	25	68	47
Aspartic acid	23	45	25	16	44	31
Glutamic acid	220	266	190	90	340	221
Cystine	9	21	17	10	26	17
Glycine	32	36	22	17	33	28
Histidine	5	19	12	6	27	14
Isoleucine	20	29	25	15	40	26
Leucine	20	22	17	9	26	19
Lysine	47	52	50	26	62	47
Methionine	5	5	5	2	6	5
Pheylalanine	21	21	14	9	28	19
Proline	53	95	52	50	110	72
Serine	41	54	62	36	53	49
Threonine	127	382	124	86	218	187
Tryptophan	1.0	4.8	1.4	1.1	4.0	2.5
Tyrosine	6	11	9	11	17	11
Valine	62	46	38	24	57	45

Table 2.18 : Effect of Some Organic Acids on the Growth and Metabolic Activity of *Leuconostoc oenos* (synthetic medium of pH 3.5).

		Time (in days)						Final pH
		2	5	9	15	22	60	
Basic medium	cells, 10 ⁶ /mL	4	10	56	57	50		3.35
	acetic acid (g/L)	0.07	0.23	0.23	0.30	0.40	0.70	
+Citric acid 3 g/L	cells, 10 ⁶ /mL	0.5	0.1	15	18	17	-	3.95
	acetic acid (g/L)	0.72	0.95	1.11	1.25	1.52	1.93	
	citric acid (g/L)	1.07	0	-	-	-	-	
+Malic acid 3 g/L	cells, 10 ⁶ /mL	3	37	70	47	24	-	3.95
	acetic acid (g/L)	0.11	0.17	0.36	0.64	1.00	1.18	
	malic acid (g/L)	1.17	0.55	0	-	-	-	
+Tartaric acid 3 g/L	cells, 10 ⁶ /mL	4	3	0.3	0.04	0.02	-	3.15
	acetic acid (g/L)	0.02	0.06	0.08	0.15	0.40	0.43	
+Glycerol 10 g/L	cells, 10 ⁶ /mL	4	5	78	70	36	-	3.60
	acetic acid (g/L)	0.13	0.17	0.36	0.68	0.72	1.00	

Fumaric acid stimulates bacterial growth at low concentrations but becomes inhibitory at concentrations above 600 mg/L. Its use for the acidification of wines to prevent the malolactic fermentation has been suggested.

The polyphenols, which are tannin compounds in must and wines, have a certain antibacterial action. The malo-lactic fermentation is more difficult to achieve in new oak barrels. The concentrations of glycerol normally encountered in wines favor bacterial growth, even if it is not used as substrate.

The strain of yeast which carries out the alcoholic fermentation also affects the malo-lactic fermentation. An inhibitory effect of a yeast strain is often attributed to the formation of SO_2 by these yeasts. It also affects the malo-lactic fermentation by non-proliferating biomass.

Sulfur dioxide in its free form as well as in its combined form with aldehydes and ketones is a potent inhibitor of lactic acid bacteria.

Stimulation of Bacterial Growth and of the Malo-lactic Fermentation

Some modifications of the fermentation have been suggested which will often permit induction of a "spontaneous" malo-lactic fermentation after a shorter or longer time interval. These are the heating of the cellar to about 19-20°C, chemical deacidification of the wine to raise its pH, and moderate sulfiting of the grapes. For quite some time one has tried to induce this phenomenon by inoculating the wine with selected bacteria. *Leuconostoc oenos* is considered to be the most appropriate species. Use of some particularly well performing strains has been proposed. The use of other species of lactic acid bacteria or pre-culture of the bacteria in the presence of yeasts has been suggested.

It is even possible to carry out the malolactic fermentation by a non-proliferating mass of bacteria which acts like a crude enzyme preparation and which is much more resistant to the conditions of the medium. Under identical conditions of ethanol concentration, malic acid concentration, SO_2 concentration and pH the kinetics of the malo-lactic fermentation depends on the wine as well as on the strain of bacteria. A mixture of various strains may well be efficacious.

Bacterial Spoilage of Wines

Spoilage by Lactic Acid Bacteria

The completion of the malo-lactic fermentation in wines does not necessarily lead to a decline of the bacterial population as the disappearance of sugar leads to a definite decline of the yeast population. If the conditions are right, that is temperature pH, and absence of SO_2 , then the bacteria can remain viable for several months in a stationary phase in the wine. The ability to assimilate organic acids and glycerol varies among strains. There are no compounds which are specific substrates for particular strains. The phenomenon is rather related to the condition of the medium and the nature of the residual substrates. Lactobacilli are frequently found in spoiled wines, but the bacteria which carry out the malo-lactic fermentation can also be agents of spoilage. Moreover, the wine contains an indigenous bacterial flora. Just those best adapted to the degradation of available substrates survive. Volatile acidity increases and the wine spoils.

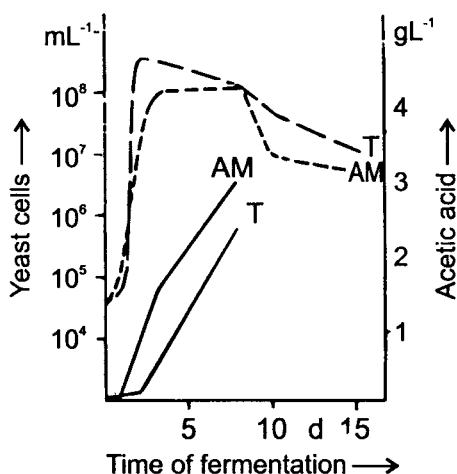


Fig. 2.12 : Production of acetic acid during the growth of a heterofermentative lactic acid bacterium

It remains nevertheless true that this experimentation confirms that for traditional fermentations the presence of malic acid results in a reduced production of acetic acid, even if a bacterial degradation of sugars occurs.

Regardless of the circumstances it is necessary to sulfite the wine appropriately once the malo-lactic fermentation has been completed in order to safeguard the wine from spoilage. Even so it may be important to determine that the metabolism of lactic acid bacteria has been inhibited during storage of the wine. Such control can be exercised by the simple quantitative determination of D(-)-lactic acid. Actually the principal product of the metabolism of these bacteria is lactic acid. L(+)-lactic acid is formed exclusively from malic acid as substrate. Both isomeric forms in variable ratios are formed from all other substrates. During the course of the alcoholic fermentation the yeasts produce several 10s mg/L of L(+)-lactic acid and less than 200 mg/L of D(-)-lactic acid. Consequently any increase in the wine of D(-)-lactic acid is an indicator of bacterial degradation of a substrate other than malic acid. The enzymatic determination of this acid is fast and precise. It allows intervention by the enologist before the continued metabolic activity of the bacteria results in an increase in volatile acidity.

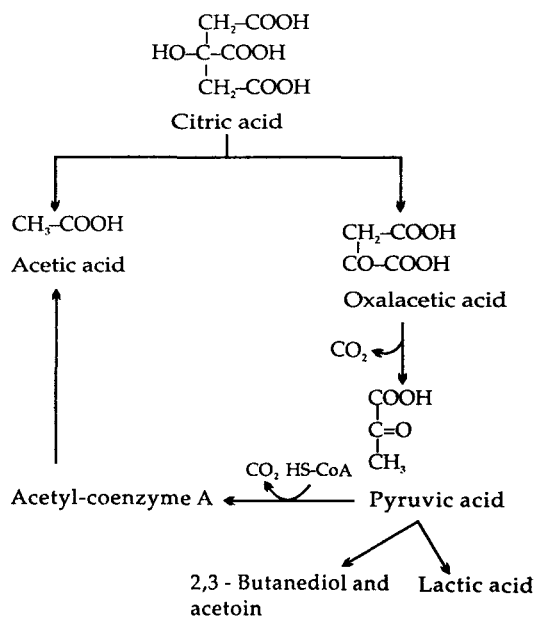


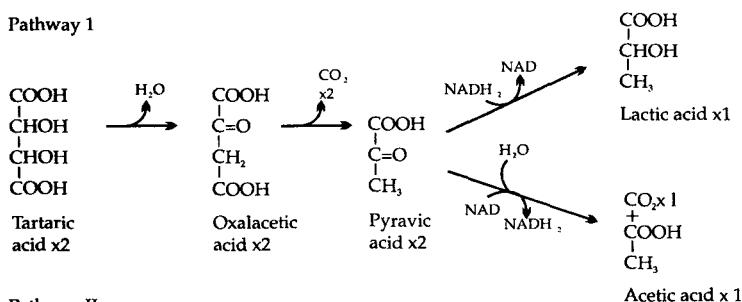
Fig. 2.13 : Metabolic pathway of the degradation of citric acid

The degradation of sugars, both hexoses and pentoses, leads to an increase in the fixed and volatile acidity of the wine; that is to the "piqure lactique".

The oxidation of citric acid by citrate lyase leads to the formation of acetic and pyruvic acid. The latter via acetyl-CoA is also converted to acetic acid (Fig. 2.13). This happens generally during or just after the completion of the malolactic fermentation. It accounts for an increase in volatile acidity which can be observed in wines after the malo-lactic fermentation. Under practical conditions 1 mol citric acid is converted to about 1 mol acetic acid.

Oxidation of tartaric acid occurs rarely (Fig 2.14). It can take place via two pathways which differ depending on whether the bacteria are homofermentative or heterofermentative. In both instances pyruvic acid appears which is secondarily converted to acetic acid. The fixed acidity decreases while the volatile acidity

Pathway 1



Pathway II

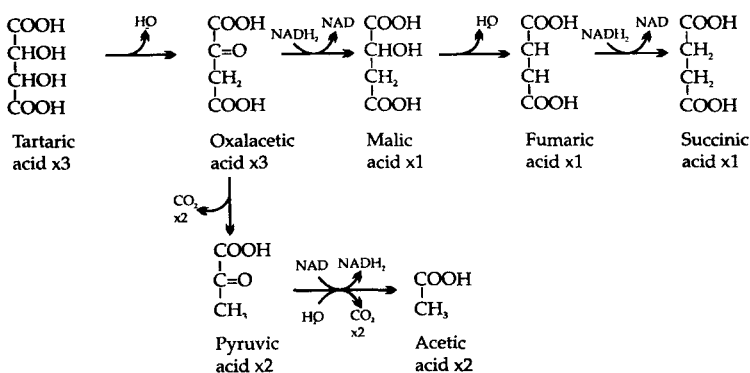


Fig. 2.14 : Metabolic pathways of the degradation of tartaric acid

increases. On tasting the wine appears flat and vinegary. The odor resembles that of fermenting sauerkraut which is due to the formation of acetoin. This is the so-called "tourne" spoilage.

The oxidation of glycerol (Fig. 2.15) leads to pyruvic acid via glycolytic pathway. Pyruvic acid is reduced to lactic acid, and to acrolein via the aldehyde of B-hydroxy-propionic acid. Acrolein reacts chemically with the polyphenols of the wine to produce a pronounced bitter flavor. Both fixed acidity and volatile acidity increase, and the odor becomes sharp. This is the "bitter" spoilage.

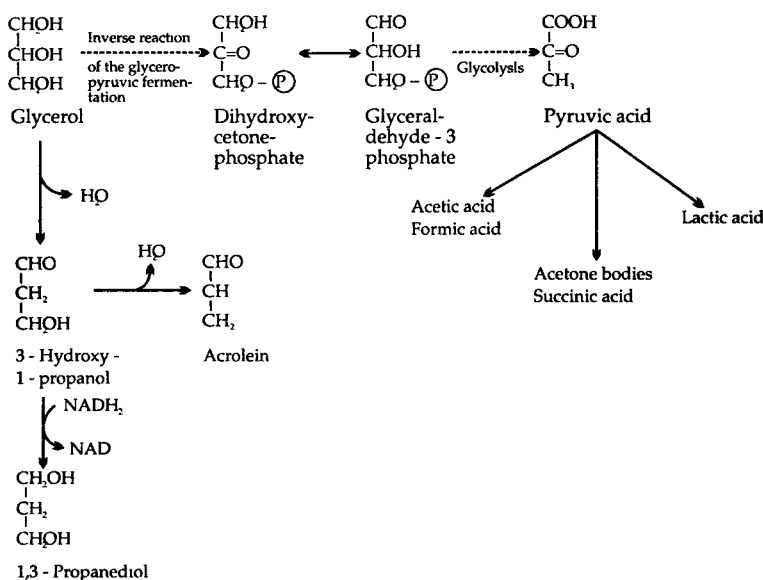


Fig. 2.15 : Metabolic pathways of the degradation of glycerol

Under conditions which are poorly understood some strains of *Leuconostoc*, *L.mesenteroides* and *L.dextranicum* (Bergey's Manual), can form a film. This film is devoid of carbohydrates and of gelatinous texture and gives the wine an oily aspect. This is the so-called "fatty" spoilage.

Recently histamine has been found in wines. The toxic material can cause serious pathological symptoms if the wine contains more than 10 mg/L.

Higher concentrations have been found in Burgundy wines. In bordeaux wines histamine can be detected in 98% of the red wines

and in 75% of the white wines. Its average concentration is 0.9 mg/L in Saint-Emilion, 3.7 mg/L in medoc, 0.2 mg/L in dry and sweet white wines (Table 2.19).

Table 2.19 : Histamine Concentrations in Grape Juices and Wines (concentrations in mg/L)

Number of Samples	Origin	Average Concentration	Minimum	Maximum
10	Grape juice, white	0.3	0	0.8
12	Grape juice, red	0.3	0.2	0.5
21	Wines St. Emilion and Pomerol	0.9	0	2.2
9	Medoc	3.7	1.4	8.9
23	Burgundy	6.4	0.4	21.0
4	Algeria	0.7	0.6	1.3
8	Diverse reds	1.3	0.3	4.8
27	Bordeaux white dry and sweet	0.2	0	0.8
6	Diverse whites, sparkling and champagnes	1.4	0.1	6.3
4	Port	0.11	0	0.4

Generally histamine is found in wines in very small concentrations largely below the threshold of toxicity. It stems in part from the grapes and in part from yeast and bacterial metabolism. Bacteria have only a slight capacity to form histamine, during their active growth and this is in no way analogous to the obligatory formation of products of the malo-lactic fermentation. The ability to decarboxylate histidine to histamine is rarely found in the bacteria of wine. In 1975 Radler reported that he found 1 strain of *Pediococcus* capable of that reaction among several 100 bacterial strains. In contrast, a non-proliferating bacterial flora remaining in the wine can develop considerable amounts of histamine (Table 2.20). This can explain the elevated concentrations of histamine which can be observed in accidental spoilage. It is an additional reason for eliminating the bacterial population of a wine by suitable sulfiting and clarification as soon as the malo-lactic fermentation has been completed.

Table 2.20 : Formation of Histamine in Wines by Bacteria in the Declining Phase (concentrations in mg/L)

	1st Wine: Histamine Conc, 0.8 mg/L		2nd Wine: Histamine Conc, 0.8 mg/L	
	Control Wine	Wine + 100 mg/L of Histidine	Control Wine	Wine + 100 mg/L of Histidine
Bacilli, homofermentative				
U 565	4.6	1.3	2.2	0.5
JF 1	-			
Bacilli, heterofermentative				
Ba 41	3.8	traces	1.2	0.3
Bc 2	-	-	2.8	0.1
F 69	6.3	0.9	2.7	0.3
CV 123	-	-	2.7	0.3
Cocci, homofermentative				
SG1	3.3	traces	3.7	0.5
CV 116	-	-	7.3	0.1
Cocci, heterofermentative				
CF 34	2.5	0.8	7.3	0.9
BR3	6.9	0.2	-	-
J0	-	-	1.3	0.9

Added wet weight of cells; 1 g/L; temperature of incubation; 30°C; time of incubation : 3 to 5 days

Spoilage by Acetic Acid Bacteria

Taxonomy, Ecology

In the field of enology the acetic acid bacteria undesirable at any stage of the wine making process (Fig. 2.16).

The acetic acid bacteria are polymorphous. Their shape is ellipsoidal or in the form of rods. They are non-motile or have ciliae. Flagella are polar with 3-8 ciliae as in *Gluconobacter* or peritrichous



Fig. 2.16 : Acetobacter

as in *Acetobacter*. The dimension varies from 0.6 to 3 μm . The cells occur as single cells, in pairs or in chains. Some species, such as *Gluconobacter oxydans* and *Acetobacter aceti liquefaciens*, produce brown water-soluble pigments and pyrones; others, such as *Acetobacter aceti xylinum*, produce cellulosic films whose composition is in all respects similar to that of plant cellulose.

Scientists have identified the lactic acid bacteria of sound grapes and of grapes infected with *Botrytis cinerea*. Among 43 isolates he found 16 *A. oxydans*, 6 *A. mesoxydans*, and 2,5-diketogluconic acid. This can explain the high concentration of bound SO_2 in musts from grapes with large populations of acetic acid bacteria.

A. aceti and *A. pasteurianus* appear with *Gluconobacter* if the grapes are slightly spoiled and constitute 45-85% of the population if they are completely spoiled. In that case acetic acid bacteria can be found in numbers equivalent to those of the yeast population, namely, 10^5 - 10^6 cells/mL (Table 2.21).

Musts from *Botrytis cinerea* infected grapes and high in sugar concentration, have a population of acetic acid bacteria which is not negligible and which can persist during the first days of the alcoholic fermentation. It is reduced to 10^2 - 10^3 cells/mL at the end of the fermentation. Progressively *Gluconobacter* is replaced by *Acetobacter* (Table 2.22).

The requirement for growth factors varies from strain to strain. The most exacting requirements are for B group vitamins: pantothenic acid, *para*-aminobenzoic acid, nicotinic acid and thiamin. The following amino acids may be required: valine, isoleucine alanine, cysteine, histidine, and proline.

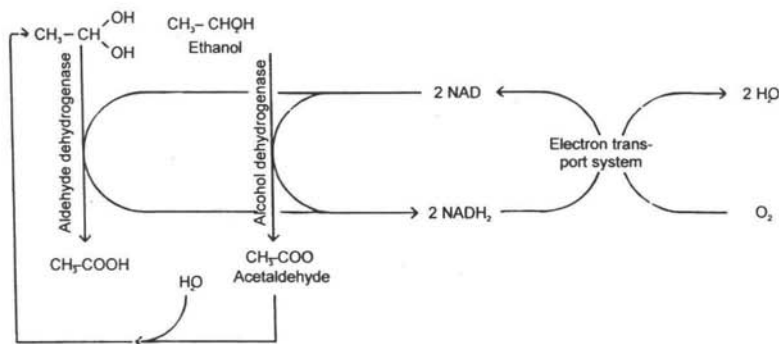


Fig. 2.17 : Metabolic pathway of the oxidation of ethanol by acetic acid bacteria

Table 2.21 : Identification of Acetic Acid Bacteria on Grapes under Various Sanitary Conditions

Samples	Bacterial Counts (per mL)	Number of Species Identified	Identification	Species	Genera
Red grapes, sound					
1.	10 ²	8	8 <i>Gluconobacter oxydans</i>	100% <i>Gluconobacter oxydans</i>	100% <i>Gluconobacter</i>
2.	80	4	4 <i>Gluconobacter oxydans</i>	100% <i>Gluconobacter oxydans</i>	
3.	10 ²	8	8 <i>Gluconobacter oxydans</i>	100% <i>Gluconobacter oxydans</i>	
White grapes					
1.	10 ⁴	20	8 <i>Gluconobacter oxydans</i>	40% <i>Gluconobacter oxydans</i>	40% <i>Gluconobacter</i>
2.	40	4	12 <i>Acetobacter aceti</i>	60% <i>Acetobacter aceti</i>	60% <i>Acetobacter</i>
			4 <i>Acetobacter pasteurianus</i>	100% <i>Acetobacter aceti</i> var. <i>orleanensis</i>	100% <i>Gluconobacter</i>
White grapes, 10 ⁵					
		14	2 <i>Gluconobacter oxydans</i>	15% <i>Gluconobacter oxydans</i>	15% <i>Gluconobacter</i>
			10 <i>Acetobacter aceti</i>	71% <i>Acetobacter aceti</i>	
Grapes, spoiled (vinegary)					
	10 ⁶	11	2 <i>A. pasteurianus</i>	15% <i>A. pasteurianus</i>	85% <i>Acetobacter</i>
			5 <i>Gluconobacter oxydans</i>	55% <i>Gluconobacter oxydans</i>	55% <i>Gluconobacter</i>
			3 <i>Acetobacter aceti</i>	22.5% <i>Acetobacter aceti</i>	
			3 <i>A. pasteurianus</i>	22.5% <i>A. pasteurianus</i>	45% <i>Acetobacter</i>

Table 2.22 : Development of the Microflora During the Alcoholic Fermentation of a Grape Must Infected by *botrytis cinerea* (Initial sugar concentration: 300 g/L)

Condition		Yeasts (10 ³ /mL) ^{a)}	Lactic Acid Bacteria (10 ³ /mL) ^{a)}	Acetic Acid (10 ³ /mL) ^{a)}	Bacteria Species	Sugar Fermented (g/L)
Initially	(1)	1.6	4.0	2000	<i>Gluconobacter</i> : 80% <i>Acetobacter</i> <i>pasteurianus</i> : 20%	0
	(2)	1.1	3.0	1500		
	(3)	5.7	6.8	1800		
	(4)	1.0	1.8	1200		
5th Day	(1)	24	2.0	80	<i>Gluconobacter</i> : 70% <i>A. pasteurianus</i> : 30%	88
	(2)	40	2.0	90		72
	(3)	44	2.7	60		96
	(4)	40	1.6	20		78
12th Day	(1)	30	0.3	<10 ³	<i>A. pasteurianus</i> : 50% <i>A. aceti</i> : 50%	170
	(2)	45	0.2	<10 ³		158
	(3)	43	0.2	<10 ³		180
	(4)	42	0.05	<10 ³		170
20th Day	(1)	12	0.01	0.6	<i>A. pasteurianus</i> : 50% <i>A. aceti</i> : 50%	220
	(2)	12	0.05	1.8		225
	(3)	9	0.01	1.1		230
	(4)	7	0.008	0.9		240

(1) Indigenous yeasts; no sulfite; (2) Indigenous yeasts; must sulfited to 10 g/hL; (3) + *Saccharomyces cerevisiae*, active dry, 10 g/hL; no sulfite; (4) + *S. cerevisiae*, active dry, 10 g/hL; must sulfited to 10 g/hL

* number of viable cells

The oxidation of ethanol by this specific route leads to the obligatory formation of an intermediate metabolite, acetaldehyde (Fig. 2.17). It requires two successive reactions, the first catalyzed by alcohol dehydrogenase (pH optimum 5), the second by acetaldehyde dehydrogenase (pH optimum 6), which are in part inducible. Acetaldehyde accumulates in the medium when the concentration of oxygen falls to low levels because its oxidation is inhibited before the oxidation of ethanol is inhibited. In addition acetic acid is a competitive inhibitor for the oxidation of ethanol.

Table 2.23 : Formation of Acetic Acid in a Wine as a Function of the Growth of *Acetobacter* under Semi-anaerobic Conditions

	<i>Acetic Acid Bacteria</i> (10 ³ /mL) ^{a)}	<i>Acetic Acid</i> (g/L)	<i>Lactic Acid</i> D (-) L (-) (g/L) (g/L)	<i>Acetaldehyde</i> (mg/L)
Initially	4	0.33	0.19 1.920	28
2nd Day	8	0.37		
3rd Day	12	0.50		
4th Day	14	0.69		
8th Day	11	0.85	0.20 1.920	52

Temperature of incubation: 19 °C pH of wine: 3.55 ethanol concentration: 12 vol%

^aViable cell counts

Some acetic acid bacteria oxidize glycerol to dihydroxyacetone and the lactate and, depending on the pH of the medium, with the formation of CO₂, acetic acid, acetaldehyde and acetoin.

Effect of the metabolism of acetic acid bacteria on the quality of musts and wine

Acetic acid bacteria are responsible for the vinegary spoilage of wines. This is characterized by (a) the oxidation of ethanol to acetic acid and (b) under anaerobic conditions the formation of ethyl acetate by esterification of alcohol and acetic acid depending on the prevailing concentrations of alcohol and acid.

Formation of acetic acid is stimulated by an increase in temperature. At 28°C it is twice as fast as at 23°C.

Ethanol at higher concentrations is an inhibitor and in practice wines with elevated ethanol concentrations rarely become vinegary. An excess of ferrocyanide from the treatment of wine inhibits bacterial growth. Phenolic compounds have a slight inhibitory action.

Wine Technology

Grapes and Corrective Measures for the Vintage

The berries consist schematically of three parts, the skin, the pips, and the pulp which is filled with juice. For most of the red grapes the anthocyanin accumulates in the skins, and only in the skins. The pips contain high concentrations of polyphenols (22% of the total polyphenols of the berry). Aromatic substances are located in the internal cells of the skins. The composition of the juice varies from berry to berry for the same grape variety. In addition each variety has its own characteristics and produces musts with higher or lower concentrations of acids and sugars. Finally, under otherwise identical conditions, each year gives vintages with a different composition depending on use of fertilizers and climatic conditions. And yet, the quality of the grape, its variety, its maturity, and its soundness determine the quality of the wine.

The ripening process can be followed by assays of acids and sugars in the juice, and by observation of the color and the sanitary state. Harvest dates vary with the grape variety and the climate (Table 2.24). In cooler climates one must protect the vineyard against mold and insect infestation in order to permit the grapes to reach physiological maturity in good sanitary condition. This is done with chemical treatment which may affect the fermentation. In warmer climates overripe grapes show a concentration of acids that is too low. It may be desirable to harvest prematurely.

Table 2.24 : Maturation Times for Some Grapes in France

	<i>Red Grapes</i>	<i>White Grapes</i>
First time span (at the same time as Chasselas)	Gamay Pinot noir	Chardonnay Melon Traminer
Second time span (12-15 days after Chasselas)	Cabernet Franc Cabernet Sauvignon Cinsaut Malbec Merlot Syrah	Altesse Chenin Muscadelle Riesling Roussanne Sauvignon Sylvaner
Third time span (24-30 days after Chasselas)	Aramon Carignan Grenache	Clairette Folle Blanche Maccabeo Ugni-Blanc

The production of some of the most prestigious, sweet white wines is based on the use of overripe grapes and the development of "noble rot". The development of *Botrytis cinerea* on ripe grapes is favored under special climatic conditions when periods of high humidity and sunshine alternate. According to the degree of infection one distinguishes the berries (pourris pleins), filled with juice with a brownish-violet skin color, and the berries (rotis) which are wrinkled, dehydrated and covered with a mycelium and conidiophores. The harvest is carried out with several selections (Auslese). One collects only those berries or bunches of grapes which have reached the threshold of the "roti" stage. The grape must contains from 260-400 g/L of sugar, is high in glycerol concentration and deacidified by a decrease in tartaric acid (Table 2.25). It contains some inhibitors of yeast and those that favor acetic acid formation, but also substances which give this type of wine a specific and greatly appreciated aroma.

During rainy fall weather the grapes which are affected by the high humidity can support a varied fungal microflora. *B.cinerea*, *Penicillium*, *Aspergillus*, and in addition *Gluconobacter* and *Acetobacter* attain numbers equal to those of the yeasts. This is the common moldiness (pourriture vulgaire) of grapes. It destroys the anthocyanins of grapes and hence their color, and causes oxidative reactions leading to the oxidative spoilage of wines, which have a disagreeable moldy taste and phenolic odor.

Table 2.25 : Composition of musts from Sound Grapes and Grapes Infected with *Botrytis cinerea*, "noble rot" (concentrations in g/L)

	Sound Grapes	Infected Grapes
Degree Baume	13°6	20°5
Reducing sugars	238	360
Total acidity (as H ₂ SO ₄)	3.92	4.02
Tartaric acid ^{a)}	5.1	4.3
Malic acid ^{a)}	2	4
Gluconic aci ^{a)}	0	1.8
Acetic acid ^{a)}	0	1.1
Glycerol	0	10.5

^a Total, i.e., free acid plus acid in the form of a salt

Natural conditions are not always optimal for the growth of vines and the ripening of the grapes. Therefore, different treatments have been authorized to compensate partially for the deficiencies of the grape must. The treatments vary somewhat from region to region. These are :

- the addition of sugar (amelioration, chaptalization). The concentration of sugar can be raised by the addition of beet or cane sugar of concentrated grape must. The sugar must be dissolved in a suitable volume of must and added to the total volume of must by mixing or pumping over. On the average 17 g/L of sugar are needed to yield 1 vol% ethanol. However, depending on some poorly understood factors (the strain of yeast and the condition of the must) this figure can vary from 16-19 g/L. Therefore, amelioration is a delicate operation. It should be practiced at the beginning of the fermentation, that is, during the phase of yeast proliferation. The rate of yeast growth is increased at moderate sugar concentrations, and the aeration which often accompanies the addition of sugar stimulates yeast growth and survival. Amelioration with sugars must be moderate, otherwise problems may arise with the fermentation because of an improper balance between the content of energy sources of the must and its nutritional capacity. An excess of sugar leads to a winy flavor which can affect the organoleptic character of the wine unfavorably. Amelioration is strictly regulated by legislation.

The use of concentrated musts, produced by vacuum evaporation, requires their deacidification before addition. In wine producing areas which are designated as "regions d'appellation d'origine contrôlée" the concentrated musts used for amelioration must come from the same region.

- deacidification. This is carried out by the addition of 3 g/L of potassium tartrate, 2 g/L of potassium bicarbonate, or 1 g/L of calcium carbonate to the must. Any of these additions reduces the fixed acidity by 1 g/L (expressed as H_2SO_4) by precipitation of potassium bitartrate. Another technique calls for use of the calcium double salt of tartrate and malate. At pH values between 4.2 and 4.5 one obtains a precipitate consisting of equimolar quantities of calcium tartrate and calcium malate. A suitable volume of must is treated in this manner, filtered and added to the rest of the acid must. Deacidification is required in cold years and facilitates eventually the final bacterial deacidification of the wine.

- **acidification.** This is mainly practiced in warm climates. It is only permitted for musts or fermenting musts. The addition of 2 g/L of tartaric acid augments the fixed acidity by about 1 g/L (expressed as sulfuric acid). It leads to the precipitation of potassium salts which lowers the pH of the wine. Citric acid should not be used because its metabolism by lactic acid bacteria results in the production of noticeable amounts of acetic acid.

Classic Fermentations

White Wine Production

White wine is the product of the fermentation of pure grape juice obtained from white grapes by extraction from white grapes. Maceration of the skins should be avoided and the must should be clarified by settling. One distinguishes two basic types, the dry, white wines and the sweet, white wines of variable sugar content.

More than all other wines the dry wines derive their character from the quality of the grapes and the techniques of the fermentation. Workers recommends that only perfectly sound grapes are used, and in cooler climates those grapes that have reached physiological maturity.

Extraction of the juice should be done rapidly to avoid oxidation and maceration. It consists of the following operations:

- **crushing.** This breaks the skins and permits the juice to run out of the pulp. Fragmentation and grinding of the berries should be avoided because they result in the formation of larger amounts of sediment.

- **draining.** This can be static when the juice runs naturally from the crushed grapes. Generally one prefers dynamic drainage by means of vibrating screens or turning movements which accelerates drainage of the must and prevents oxidation.

- **pressing.** This permits extraction of the must remaining in the pulp. Vertical presses yield juice with little sediment because it has been filtered across a cake of pulp. But the system requires several successive pressings after crumbling and turning over of the pulp. This is a lengthy operation and may lead to oxidation. The operation is more readily automated with horizontal presses in which the pressure is not as high. Nevertheless, the press juice is strongly aerated pneumatic presses permit a less damaging

extraction, with better distribution, and better maintenance of the integrity of the grapes. Continuous presses are best suited for larger operations. But this rapid and violent extraction pulverizes the press cake.

Regardless of the method of extraction the free run juice and sometimes the first press juice alone produce quality wines. For white wine production a selection of the juice is mandatory. Pressing of intact grapes without crushing yields musts which are easier to clarify and have a larger volume of clear must with improved fermentability.

The use of pectic enzymes in crushed grapes permits faster extraction of a larger volume of must.

treatment with bentonite is used to remove the proteins of the must and to prevent after-precipitation that is sometimes called "protein floc". Bentonite is an adsorbing clay. It is usually added before the fermentation to the clarified must at a rate of 60-100 g/hL. After the fermentation it sediments without significantly increasing the volume of lees. This treatment also gives the wine a certain aromatic cleanness.

The clarified must is stored in casks or more often in tanks. Fermentation is carried out anaerobically at a temperature below 20°C. The yeast synthesizes at the relatively low temperature larger quantities of volatile substances which constitute the secondary aroma of wines. The addition of chemical fermentation activators and suitable inoculation with yeasts can prevent the fermentation difficulties which are frequently encountered with highly clarified musts.

The fermentation is considered complete when the residual sugar is less than 2 g/L. At this point SO₂ should be added at 6-8 g/hL and the wine should be clarified as quickly as possible by filtration or centrifugation. Prolonged storage on the yeast containing lees can cause the development of sulfhydryl compounds of disagreeable odor. If a malo-lactic fermentation is desired, the wine remains on the lees without sulfiting until the malo-lactic fermentation has taken place. The wine is then sulfited and filtered.

Among the sweet white wines one distinguishes often the semi-dry wines, mellow wines, and sweet wines without a proper range

of residual sugars for each type. In France such wines can be made by arresting the fermentation before all of the sugar has been fermented. The arrest is spontaneous or through the addition of SO_2 . It presupposes the availability of grapes particularly high in sugar concentration, possibly induced by overripening caused by infection with *Botrytis cinerea*.

The extration of the must requires the same precautions to avoid oxidation and maceration. For grapes infected with *B.cinerea* the dehydration of the berries and the senescence of the tissues makes the draining of must useless and pressing difficult. Must clarification is practically impossible because of the high viscosity of the must.

The first press juices obtained with horizontal or vertical presses yield a must rich in sugar but with good fermentability and whose fermentation forms only little acetic acid. The last press juices yield musts which are difficult to ferment and high in glucan concentration and in polysaccharides. Such musts often inhibit yeast growth and lead to the formation of acetic acid.

The moderate addition of sulfite (5 g/hL) prevents bacterial contamination and facilitates the fermentation by partially inactivating the yeast inhibitors secreted by *B.cinerea*. The addition of activators accelerates the fermentation in the beginning but has little effect on the total quantity of fermented sugar. Yeasting with a combination of selected strains, *Saccharomyces bayanus* and *S.rosei*, seems most useful. For this type of must the spontaneous arrest of the fermentation after 4-5 weeks is almost the rule. The wines contain higher concentrations of aldehyde and keto compounds and bind more sulfur dioxide. Considerable quantities of SO_2 are, therefore, required for its conservation.

In musts of somewhat lesser sugar concentrations, the fermentation can be stopped by massive use of SO_2 which permits 80 mg/L of free SO_2 to remain in the wine. The success of this operation is better assured if the yeast population is partially eliminated by racking. The same results can be obtained by heating or sterile storage.

Mellow wines can be obtained more easily by sugaring dry wines, that is, by addition of fresh must or concentrated must at the time of bottling.

Red Wine Production

Red wine production is based on maceration, i.e., the prolonged contact of the must with the solid parts of the grapes. The alcoholic fermentation is accompanied by the maceration of skins and seeds, and sometimes of the stems, which impart to red wine their color and their tannin content. After complete fermentation of the sugar and a suitable time for maceration the wine is drawn from the pomace and eventually undergoes a malo-lactic fermentation.

Traditionally the processing of grapes comprises two operations which are usually mechanized and often carried out simultaneously:

- crushing is carried out to expose the pulp. It favors maceration and consequently the dissolution of color and tannins from the skins. By partially homogenizing the medium, it activates the fermentation and permits transfer of the mass by pumping.
- destemming consists in the separation of the stems from the berries. The stems give the wine an astringent, herbaceous taste, and their removal yields more subtle and refined wines. In some instances destemming is not recommended.

The crushed and destemmed grapes are sulfited to protect against oxidative, enzymatic processes and against microbial spoilage.

The tanks of very large volume and preferably made with stainless steel are closed. This prevents evaporation, and the proliferation of acetic acid bacteria in the cap. The cap consists of the insoluble portions (skin, seeds, pulp particles) and is carried to the top of the fermenter by the formation of carbon dioxide bubbles.

The fermentation is carried out at a relatively elevated temperature between 25 and 30°C which facilitates color extraction. It is generally faster than white wine fermentations. The initial yeast population is more numerous, and the fermenting medium is richer in nitrogenous and activating substances. All this permits a 10 fold multiplication of the yeast population with the danger of overheating of the fermenting must which would quickly inhibit the yeasts. Cooling of the fermenter is recommended. Equally, the fermentation is stimulated by aeration which results from the pumping of the must (drawn from the bottom of the fermenter) over the cap. This is done during the phase of yeast proliferation and

stimulates cell multiplication and cell survival. It also increases color extraction by the passage of the pumped-over must through the cap.

The drawing of the wine takes place after transfer from the fermenter to large casks. The pomace is pressed. The drawn wine and the press wine are placed into fermenters to complete the fermentation, that is, to convert the last grams of sugar to ethanol, and for the malo-lactic fermentation. The wines are then stabilized by the addition of 15 to 25 mg/L of SO_2 . The press wine with a slightly lower alcohol content has a higher concentration of anthocyanin and tannin and also a larger bacterial population. The drawn wine ("grand vin") may or may not be mixed with the press wine in variable ratios depending on their relative qualities.

The grapes are not crushed but preserved under an atmosphere of CO_2 for a period of time which varies with the temperature. Enzymatic degradation of the sugar in the intact berries forms 1.5 to 2.5 vol% ethanol. Malic acid is partially metabolized, and the juice is enriched in nitrogenous compounds, minerals and polyphenols. The pectins of the plant cell wall and the aromatic compounds of the grape skin diffuse into the pulp. These transformations take place only in intact grapes. About 20% of the berries are crushed by the weight of the mass of grapes and undergo an anaerobic yeast fermentation. After draining the drawn wine and the press wine are mixed for the complete fermentation of residual sugars. the malo-lactic fermentation follows quickly.

This technique yields wines with a subtle aroma and a remarkable intensity of the aromatic character. It is suitable for the fermentation of neutral grape varieties and the production of "semi-fine" wines which are consumed shortly after production.

A third technique, thermovinification, consists in heating the intact grapes or more commonly the crushed grapes to about 75°C. Heating must be sufficiently high to "denature" the skins and fast enough so that the temperature of the pulp remains at about 30°C. Thus result can be obtained by several techniques and serves the accumulation of anthocyanins, tannins, polysaccharides, organic acids, nitrogenous materials and aromatic compounds in the must. The alcoholic fermentation is rapid and complete, and so is the malo-lactic fermentation. The fermentation of a single must permits a reduction in the volume of fermenters and automation of the operations (elimination of additional tank to tank transfers). The

fermentation with this type of maceration produces highly colored and aromatic wines.

The great red wines as well as some white wines are stored for two years in barrels. Four times each year they are decanted to eliminate lees as well as excess dissolved CO_2 . Each of these operations is followed by readjustment of the SO_2 concentration. Frequent additions of wine are required to avoid a head space in the barrels which would favor growth of acetic acid bacteria.

During storage of the wine the color is modified by the combination of anthocyanins and tannins and their condensation. This reaction is activated by oxygen. Aromatic components are also transformed. Some esters formed during the fermentation are hydrolyzed, the fruity character disappears while tannin like odors are formed. The wine extracts aromatic elements from the wood lending it a vanilla-like odor which is greatly appreciated.

But successive decantations do not suffice in clarifying the wine, and, particularly in stabilizing its color. Therefore, the wine is fined before bottling. The fining process consists of the addition of proteins in the form of gelatin, globulin, casein, egg white, etc., or bentonite. The reaction of the protein with the tannins and the coloring matter of the wine causes a flocculation. The sediment carries with it other suspended impurities of the wine. Filtration completes the fining operation. Red wines which have not been fined deposit considerable amounts of colored sediment after several months' storage in the bottle.

Bottling is actually mechanized. It requires perfect hygiene both with regard to the bottles and the bottling machine. Sampling for microbiological control on a statistical basis is recommended in order to reveal contamination by yeasts or bacteria. Closure with cork remains the dominant method for fine wines. The cork must be of good quality to avoid dripping or a corky taste. During storage in the bottle additional modifications of the bouquet of the wine occur. Some wines require several years storage in the bottle to reach the height of their quality.

Biological Stabilization of the Wine; the Role of Sulfur Dioxide and Sorbic Acid

Wine with its complex chemical composition can undergo some reactions which lead to the formation of haze or deposits, such as

oxidative, ferric, cupric, or proteinaceous precipitates as well as the precipitation of bi-tartrate or coloring matter. Today one can foresee such problems and one has the means to prevent them.

With regard to the development of microorganisms the wine is better protected than other food products because of its acidity and the presence of alcohol. Nevertheless, complete biological stabilization requires the use of chemicals, such as the addition of SO_2 or sorbic acid, or physical processes such as pasteurization or hot filling of bottles.

In a general way the wine is protected by SO_2 throughout its production stages. This antiseptic is universally employed in cellar practice, usually in the form of sulfurous acid solutions, for the treatment of musts and the conservation of wine. It acts first of all by inhibiting the oxidative enzymes of grapes, and specifically tyrosinase and lactase in moldy grapes. It also acts as an inhibitor of microbial growth. Depending on its concentration it inhibits yeast and bacterial metabolism temporarily or permanently. In musts and wines SO_2 combines partially with carbonyl compounds. The rest constitutes free SO_2 . The equilibrium between free SO_2 and bound SO_2 varies with the pH, the temperature, and the concentration of carbonyl compounds of the medium. Free SO_2 is present either in the form of free sulfurous acid (H_2SO_3) or the ionized bisulfite (HSO_3) depending on the pH of the wine. Only H_2SO_3 acts as inhibitor of microbial metabolism and growth. Its proportion depends on the pH. It is 10% at pH 2.8 and 1% at pH 3.8. Consequently, the effectiveness of sulfiting depends greatly on the pH.

One of the principal combination compounds is that of SO_2 and acetaldehyde. The presence of pyruvic and α -ketoglutaric acid can also threaten the biological stability of the wine. In a wine with 100 mg/L of free SO_2 each of these two acids, respectively, can bind 60 and 35 mg/L of SO_2 . The must from grapes infected with *Botrytis cinerea* and acetic acid bacteria contains ketogluconic acids which can bind SO_2 . Besides, the lack of thiamin in these musts does not permit decarboxylation of the keto acids by yeasts. This results in excessive concentrations of combined SO_2 in such wines. While only free SO_2 acts as an inhibitor of yeasts, it is possible that the sulfonates increase this inhibition. Absorbed by the cell, SO_2 combines with intracellular acetaldehyde as it is formed and blocks

the alcoholic fermentation. It also denatures proteins by reducing -S-S- bridges. In moderate concentrations the action of SO_2 is not irreversible. It merely delays the onset of the fermentation which is the reason for its use in the clarification of musts by settling (for white musts) and for the protection of musts.

The effect of SO_2 is progressive. In a rapidly fermenting grape must with 10^7 cells/mL of *Saccharomyces cerevisiae* one finds that 90% of the cells are viable 5 hours after the addition of 250 mg/L of SO_2 . After 24 hours only 10^3 cells/mL remain viable. The fermentation appears to have stopped. However, one observes a slow liberation of acetaldehyde into the medium. After a few days the SO_2 is completely in the combined form and the fermentation starts again.

Sensitivity to SO_2 as an antiseptic varies with the microbial species. Besides some yeasts can form SO_2 during the fermentation.

With regard to the lactic acid bacteria it has long been agreed that sulfite protects against these bacteria only while it is present in its free form, that is, roughly between the time of addition and the start of the fermentation. Most authors attribute only a negligible bactericidal effect to combined forms of SO_2 .

SO_2 bound to acetaldehyde or pyruvic acid is also bactericidal, although 5 to 10 times less so than free SO_2 . The efficacy of bound SO_2 depends greatly on the bacterial culture and the pH.

The addition of SO_2 to the grapes seems useless from the microbiological point of view if one can be assured of an alcoholic fermentation free from problems, and particularly if one desires a rapid start of the fermentation by inoculating with yeast. Nevertheless, some precaution seems necessary to guard against some accidental problems with the fermentation and against ultimate contamination problems. SO_2 must be used with good judgement; in quantities sufficient to prevent formation of the "pique lactique", but insufficient to prevent the later growth of bacteria and the malo-lactic fermentation. The grapes must only be sulfited moderately if a malo-lactic fermentation appears desirable. Acetic acid bacteria are highly sensitive to SO_2 .

Sorbic acid is a fungicide whose use has been permitted. The dosage is limited to 200 mg/L. Its inhibiting effect on yeast depends on its concentration, and the number of yeast cells, as well as on the

Table 2.26 : Direct Bactericidal Action of Anhydrous SO₂ on a Suspension of Lactic Acid Bacteria in a Phosphate Buffer at Different pH Values (values shown are percent of cells remaining viable in relation to the population of a control medium).

Species	pH	Free SO ₂ (mg/L)			SO ₂ -Pyruvic Acid Addition Compound (mg/L of SO ₂)			SO ₂ -Acetaldehyde Addition Compound (mg/L of SO ₂)		
		5	30	100	5	30	100	5	20	100
<i>Leuconostoc gracile</i>	3.0	0	0	0	68	33	14	67	63	55
	3.2	0	0	0	87	56	42	89	79	67
	3.4	0	0	0	90	70	62	95	87	68
<i>Pediococcus cerevisiae</i>	3.0	7	0	0	25	0	0	100	0	0
	3.2	52	0	0	85	0	0	76	48	1
	3.4	60	15	0	85	75	25	100	75	50
<i>Lactobacillus hilgardii</i>	3.0	48	5	0	86	13	6	50	35	5
	3.2	49	6	1	88	107	79	74	37	32
	3.4	70	8	7	96	75	54	84	68	35
<i>Streptobacterium</i>	3.0	70	10	1	83	66	53	98	96	89
	3.2	60	23	1	96	88	70	88	76	85
	3.4	66	33	1	86	82	60	96	85	83

conditions of the medium such as the concentration of ethanol, the pH, and the presence of SO_2 . It does not accumulate within the yeast cells and only a small portion of the sorbic acid is degraded by a process which is not yet understood. In its presence the yeasts have a tendency to form a pseudomycelium. These observations have led to conclude that a complex formed by sorbic acid with thiol groups of the cell wall is responsible for its inhibiting activity. Sorbic acid, at the concentrations used in wine has no effect on lactic or acetic acid bacteria. Its metabolism by lactic acid bacteria leads to the formation of hexanediol with an odor resembling that of geraniol. For this reason one can use this antiseptic only together with SO_2 . Its use in red wines is not recommended.

Hot bottling (thermolisation) is a more practical process. The wine is sterilized by heating and filled into sterile bottles. The temperature of pasteurization need not be particularly high because of the acidity and the ethanol content of wines.

Two techniques are actually practiced which differ in their time/temperature relationship:

- hot bottling: the wine is heated to 50°C during a longer or shorter time period. It circulates at this elevated temperature in the bottling apparatus and at which it is actually filled into the bottles.
- flash pasteurization: the wine is heated for several seconds to $80\text{--}85^\circ\text{C}$ and then filled into bottles. This treatment kills a certain proportion of the microbial population, but microbiological control is mainly suitable for the stabilization of young red wines and mellow white wines of intermediate quality.

Special Processes

Sparkling Wines

These are effervescent wines produced by means of two successive alcoholic fermentations. The fermentation of the must yields a base wine which undergoes a "secondary fermentation" either spontaneously or after addition of sugar and inoculation with yeast.

In France sparkling wines with an "appellation d'origine controlee", such as Blanquette de Limoux, Clairette de Die, Saumur, etc., become sparkling by fermentation in the bottle at the geographical area of their "appellation". The designation "methode

champenoise" may be used if the wines is kept in the bottle on the yeast lees and for a period of at least 9 months. For the more ordinary sparkling wines the secondary fermentation is carried out in a closed vessel.

But the most prestigious of such wines is champagne. It comes from a well defined production area and from specified grape varieties. It is processed in the circumscribed Champagne viticultural area, and is kept for at least one year in the bottles.

The wine of Champagne is produced from two red varieties, the Pinot Meunier and the Pinot noir, and from one white variety, the Chardonnay. One must, therefore, produce a white wine from mostly red grapes. Hence everything possible is done throughout the vinification to keep the red color of the skins from diffusing into the must and to avoid oxidative processes. The grapes are harvested prior to physiological maturity, and spoiled grapes are discarded. They are transported in wicker-baskets so that the juice which sometimes drips from crushed grapes can run out.

Extraction of the must is done promptly to avoid maceration, and there is no crushing operation. The intact grapes are pressed in vertical hydraulic presses or in horizontal presses. The different must fractions are fermented separately. The first two fractions from the press, the "cuvee" is used for the production of quality champagne, the following fractions, "first cut" and "second cut", yield wines of lesser quality. The last fraction from the press is used as distilling material. The musts are clarified by settling with the addition of 5 to 8 g/hL of SO_2 . The elimination of proteins requires a slight addition of tannin. For this purpose one uses gelatin or isinglass in preference to bentonite, which ultimately reduces bubble retention. The low pH of the musts, about pH 3, favors flocculation.

The musts after amelioration are fermented at a temperature of 20°C or below in large fermenters. Until the past few years the opinions of champagne producers regarding the desirability of inducing a malolactic fermentation differed somewhat. This type of wine requires a certain freshness that is associated with an acidity of 5-6 g/L (expressed as H_2SO_4). But the modifications of the composition of nusts which have occurred, particularly the increase in pH following higher potassium levels, make the wines more receptive to the development of lactic acid bacteria.

The wine free from malic acid and with an ethanol concentration of 11-12 vol%, is drawn off, clarified, and a sufficient quantity of sucrose syrup is added. The amount of sugar syrup required is based on the pressure desired after the bottle fermentation.

Champagne always contains a population of dead yeast cells which autolyze progressively. This explains the proteolytic activity which can still be detected in wines which are several years old. The photolysis of sulfur containing amino acids by light passing into the bottles and activated by riboflavin of the wine may be responsible for the "gout de lumiere" which has recently assumed particular importance.

Another so-called "natural" or "rural" method consists in a limitation of the primary fermentation of the must. The wine which still contains about 25 g/L of sugar is then bottled. The premature stoppage of the primary fermentation is achieved by the progressive depletion of the nutrients in the fermenting wine. This is carried out by the growth of successive yeast populations which are periodically removed by a series of filtrations or by settling in the cold. The secondary fermentation is carried out in the bottles generally without inoculation. It lasts for a longer or shorter period of time depending on the temperature. For the production of sweet wines by this process there is a serious problem with the arrest of the secondary fermentation. It is difficult to master this technique. It is difficult to master this technique. It can be assisted by the use of yeast with a lesser alcohol producing capacity.

Other wines are called crackling, "spritzy" or "petillant". They differ from sparkling wines generally by the lower pressure of the bottle fermentation obtained by a lesser quantity of added sugar.

Finally there are the carbonated wines. They are made by saturation of the wine with CO₂. Their quality is not comparable to that of the other wines mentioned above.

Sherry and Port

Sherries owe their characteristic to an aging process based on the development of a particular yeast flora. The most prestigious sherries are produced in Southern Spain not far from Xeres de la Frontera and Sanlucar de Barameda. They are made from three principal grape varieties of Jeres, Palomino Fino and Pedro Ximenes. The original technique is that of the "solera".

In the traditional method the grapes prior to pressing are exposed to the sun ("soleo") for a day. During this time the concentration of sugar and tartaric acid increases and malic acid is partially degraded. The must is then extracted under light pressure. The juice from successive pressings is collected separately and fermented in relatively small volumes (500 to 600 L) which facilitates temperature control. Before that, the musts are sulfited and calcium sulfage is added. This last mentioned operation (plastering) facilitates the ultimate clarification of the wine.

The wines are kept on the lees until spring. The wines selected for aging are drawn off, fortified with ethanol to 15 vol% and kept with a large surface exposed to the air. A yeast film, "flor", develops spontaneously on the surface. It consists essentially of *Saccharomyces beticus*, *S. cheresiensis*, *S. montuliensis*, *S. rouxii* as well as *Hansenula*, *Zygosaccharomyces* and *Pichia*. A second selection by tasting determines the wines which will undergo biological aging, the "crianza de flor". Various strains of *S. bayanus* multiply on the surface using ethanol as substrate. Growth is inhibited by too high a concentration of SO_2 , the presence of residual sugar or a lack of nitrogenous compounds or biotin. Sufficient aeration and a temperature of 18-20°C are required. The incompletely filled barrels are arranged in stages ("escalas"). During aging the wine is transferred and mixed from barrel to barrel three or four times each year. This achieves a certain homogeneity of age and character at the time wine is bottled.

Some biochemical transformations take place during aging. Ethanol is partially oxidized to acetaldehyde, a reaction which is catalyzed by alcohol dehydrogenase. Flor yeasts contain ample amounts of this enzyme. About 400 mg/L of acetaldehyde are formed during growth depending on the alcohol concentration of the wine. It is less active at ethanol concentrations of 7 vol% and inhibited at ethanol concentrations above 15 vol%. Glycerol is also partially degraded. The base wine contains 7-8 g/L of glycerol, and after 8-10 years of aging its concentration is only about 2 g/L. Malic acid disappears. Acetic acid is assimilated particularly during the growth phase. The concentration of amino acids decreases; mainly that of proline, leucine, valine, and phenylalanine. The concentration of acetoin and 2,3-butanediol increases. One also observes the formation of lactic acid by both bacteria and yeasts. The formation of diethyl acetal together with acetaldehyde participates

in the characteristic aroma. The flavor is slightly bitter and resembles that of almonds. The long conservation in a reductive atmosphere also assures the pale yellow color which is desired.

Wines of secondary quality are fortified with ethanol to 18 vol%. They are aged by chemical oxidation to yield the "oloroso" sherries; the inferior quality is called "rayas".

Before bottling the wines are clarified with gelatin or powdered blood.

Actually acetaldehyde is formed immediately on contact with the yeasts but does not diffuse well into the medium. The process can be accelerated by mixing and aeration. Inoculation of the fortified wines with selected yeasts encourages rapid production of acetaldehyde and avoids bacterial contamination.

Port wine is made by the fortification of fermenting grape must with brandy. It is produced in the Douro region of Portugal from red and white grape varieties. Depending on the sugar content one distinguishes extra dry, semi-dry and sweet wines. The color varies from yellow to deep red.

The grapes are crushed without prior removal from the bunches. Crushing is done by more or less mechanical processes. The skins must be broken to permit diffusion of color and aroma. The fermentation is stopped by addition of brandy of 77-78% ethanol concentration by volume in order to fortify the wine to 18 vol%. Aging is carried out in barrels of 550 L capacity which are not filled completely and lasts 4 to 8 years. Port wines of high quality are still kept for 2 years protected from air before they are bottled. These wines are characterized by their rich aroma.

Brandy

Brandies are produced by distillation of wines. They have different characteristics depending on the soil, the climate, and the grape variety, as well as on the methods used in producing the wine and on the technique of the distillation. The best known brandies of France are Cognac and Armagnac which are produced by distillation of white wines from strictly defined geographical areas.

The grapes are crushed and pressed in vertical or horizontal presses. They must be fermented in cement tanks at a relatively high temperature, 25°C, without prior sulfiting or clarification. The use of sulfur dioxide is not permitted for these fermentation for the

following reasons: (a) passage of SO_2 into the brandy leads to the formation of sulfuric acid which can lower the pH too far; (b) the combination of acetaldehyde and SO_2 has the properties of an acid sulfonate, that is, of a strong acid which corrodes the copper of the distilling columns; (c) the presence of sulfur dioxide or bisulfite favors the reaction of ethanol and acetaldehyde to acetal catalyzed by H^+ ions. (d) the presence of SO_2 favors the formation of acetaldehyde during the alcoholic fermentation and lowers the aromatic quality of the Cognac.

The fermentation of the must is spontaneous. An ecological study of the yeasts of the Charentes shows that the following species are present in the must and during the fermentation, besides the dominant *Saccharomyces cerevisiae*; *S.uvarum*, *S.rosei*, *S.capensis*, *S.chevalieri*, *S.globosus*, and quite often *Saccharomycodes ludwigii*. The musts have low sugar concentrations—less than 170 g/L-, low concentration of tannic substances, and high concentrations of malic acid. They yield wines of high acidity and relatively low concentrations of ethanol quite suitable for the ultimate production of brandy. During storage prior to distillation the wine is simply protected by its high acidity and its alcohol content.

The wines are kept on the lees until they are distilled. The heavy lees consisting of sediment, broken particles, pits, or pulp are eliminated. In contrast the light lees consisting essentially of yeast maintain a reducing environment and protect the wine against "maderisation".

The wine is distilled with the yeast lees.

During storage there is a significant loss of volume due to evaporation, mainly during the first year.

The concentration of alcohol is reduced by 6-8% in a period of 15 years. The dissolution of soluble oak compounds (tannins, polyphenols, lignins, proteins, pectins and minerals) can be seen in a change of the color of the brandy. The pH is lowered from 5 to 3.5 in 50 years. During the first years acidity increases, acetal is formed, and extracted tannins are progressively oxidized which intensifies the color of the brandy. Hemi-celluloses are hydrolyzed, lignins and esters are alcoholized, and a vanilla-like or flowery odor appears. Between 10 and 30 years of storage the aroma is concentrated by evaporation of water and ethanol. The taste is sweeter because of

the lower alcohol content because of the formation of sugars by hydrolysis of the hemi-celluloses. The concentration leads to a stroger aroma (Table 2.27).

Armagnac is produced in France from the grape varieties Bacco blanc, Ugni blanc, Colombard and Jurancon. Fermentation is traditional. The wine is not clarified and neither sulfur dioxide nor other enological materials are added. Its ethanol concentration is 8-9 vol%, and it is stored on the yeast lees which are removed before distillation. The distillation is carried out by one of the following procedures: (a) continuous distillation, (b) discontinuous distillation (redistillation). The Armagnac type still is made of copper, with continuous feed and with 2 or 3 heating vessels in series (vertically). The alcohol concentration must not be higher than 72 vol%. Armagnacs produced by

Table 2.27 : Average Composition of Volatile Substances in Cognac (mg/L, 300 samples).

Acetaldehyde	32.4
Diethyl acetaldehyde acetal	12.6
Methanol	413
1-Propanol	226
Isobutylalcohol	813
1-Butanol	trace
2-Butanol	trace
2-Methyl 1-butanol	341
3-Methyl 1-butanol	1584
1-Hexanol	24.3
Phenyl ethanol	36.4
Ethyl acetate	268
Ethyl propionate	2.4
Ethyl butyrate	1.9
Ethyl caproate	10.8
Ethyl caprylate	13.6
Ethyl caprate	35.2
Ethyl laurate	36.8
Ethyl myristate	11.8
Ethyl palmitate	6.7
Ethyl lactate	268
Isoamyl acetate	8.2
Hexyl acetate	1.2
Phenethyl acetate	0.1
Diethyl succinate	4.8

double distillation have a higher concentration of higher esters, particularly ethyl caprylate, caprate and laurate. One can determine the method by which a young Armagnac has been distilled by its concentration in butanediol and ethyl laurate (Table 2.28).

Armagnac is aged in casks made from the peduncular oak which is richer in tannins than the oak used for the agins of conyac. During aging the hemi-celluloses and other polysaccharides are partially hydrolyzed and yield various sugars like glucose, xylose,

Table 2.28 : Average Composition of Armagnacs

	<i>I.^{a)}</i> (mg/L)	<i>II.^{b)}</i> (mg/L)
Volatile acids (as acetic acid)	300– 400	150– 200
Aldehydes as acetaldehyde	50– 80	50– 80
Furfural	4– 10	15– 30
Esters	500– 1000	500– 1000
Higher alcohols	3000– 4200	3000– 4200
Simple compounds		
Acetyldehyde	50– 70	50– 70
Ethyl acetate	400– 750	400– 750
Methanol	500– 600	500– 600
2-Butanol	0– 20	0– 20
Propanol	350– 450	350– 450
Isobutylalcohol	700– 1000	700– 1000
1-Butanol	0– 20	0– 20
Isopentylalcohol	2100– 2700	2100– 2700
Hexanol	6– 35	10– 33
Phenyl ethanol	20– 80	9– 32
Ethyl lactate	100– 500	100– 500
Ethyl caprylate	5– 50	8– 100
Ethyl caprate	2– 4	6– 140
Ethyl laurate	2– 10	5– 70
Ethyl myristate	1– 3	4– 20
Acetion	5– 15	2– 8
Butanediol	20– 30	0.5– 3

^a I. distilled once

^b II. distilled twice

arabinose, and fructose, which sweeten the taste. The tannins are oxidized and polymerized which lessens their astringency.

The volatile compounds in brandies are derived both from the grapes and the fermentation. They are concentrated by distillation and modified during aging. For certain grape varieties they reach the greatest intensity if they are grown in relatively cool districts.

The bouquet of brandy consists of a complex mixture of higher alcohols, esters, fatty acids, aldehydes, acetal, and volatile acids

produced during the fermentation. Nevertheless, it seems that characteristic differences are found for higher alcohols depending on the fruit used in production.

In brandies at 50 vol% ethanol the higher alcohols are generally within the range of 650 to 1000 mg/L, depending on the composition of the must, the microorganisms of the fermentation, and the conditions employed during vinification. The principal higher alcohols are 3-methyl-1-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and n-propanol. At high concentrations they have an unfavorable effect on the bouquet of the brandy.

The following esters are the major contributors to the aroma: ethyl, hexyl and isopentyl myristate; monoethyl succinate; capryl enanthate ethyl oleanoate, and ethyl phenylcaproate. The presence of the last named compounds can be considered a sign of brandy quality.

The concentration of fatty acids in brandies is directly correlated with the yeast biomass in the wine at the time of distillation. Fatty acids of lower molecular weight are excreted by the yeasts into the wine, but those of higher molecular weight pass into the liquid at the time of distillation. Their concentration in brandies depends on the production techniques used. The presence of propionic and butyric acids is undesirable. Caproic, caprylic, pelargonic, lauric, myristic and stearic acids are found in Cognac and play an important role in its aroma.

Acetaldehyde is the predominant carbonyl compound, but it is largely eliminated by removal of the heads. Furfural (pyromucic aldehyde) is formed during the distillation in the presence of lees containing lignin compounds. Other aldehydes are formed during aging. Acetaldehyde is formed by oxidation of ethanol and some aldehydes are extracted from the wood of the barrels. Diethyl acetal which is present in all brandies is formed by condensation of ethanol and acetaldehyde. During the aging of brandy in barrels some lignin is alcoholized. Oxidation of the compounds formed results in the formation of aldehydes of the vanilla type which play a major role in the aroma of brandies (Amerinc *et al.*, 1980).

The quality of brandy and notable that of Cognac is determined by a complex of factors: nature of the soil and subsoil; grape variety; management and density of cultivation; sufficient ripening of

grapes for a minimum sugar concentration; vinification, storage of the wines; distillation, storage of the brandies and aging.

Brandies are produced in all viticultural regions of the world. A respect for the rules derived from a knowledge of viticulture and enology will permit an improvement of their quality.

3

Beer

Beer is the beverage obtained by the alcoholic fermentation of the water extract of malted cereal grain to which hops have generally been added. The malted cereal is most generally barley; other starchy material may be used with the malted barley.

Man's Earliest Brewing

No doubt the first thinking man, *Homo sapiens*, was at the seat of bread making and beer fermentation. His reasoning power and intelligence allowed him to recognize the necessary steps of malting his grain. This allowed the parallel development of baking and brewing and gave early man two basic foods from his cereal grains.

Ancient Aryans produced a beer-like beverage called *sura* from grains as early as 2000 B.C. As did many of the ancient civilizations, they attached supernatural powers to fermented beverages and viewed drunkenness as a religious experience. Their drinking was largely ceremonial, frequently accompanied with offerings to their gods.

Beer Types in the World

Many of the old brewing centers developed particular beers which were distinctive and came to be identified with the locality. Distinctiveness of the beers sometimes was the result of a natural phenomenon such as the mineral content of the water or it could have been due to the brewing practice(s) or material(s) employed in a particular area.

Classical Beer Types

Table 3.1 lists some of the historically distinctive beers that are still popular and gives some facts about them. The beers are divided into three groups, arranged according to their general character.

Table 3.1 : Classical Beer Types Brewed in the World

Type	Character	Origin	Alcohol	Flavor Features
<i>Bottom Fermented</i>				
Münchener	Lager/ale	Munich	4-4.8	Malty, dry, mod, bitter
Vienna (Marzen)	Lager	Vienna	5.5	Full bodied, hoppy
Pilsner	Lager	Pilsen	4.5-5	Full bodied, hoppy
Dortmunder	Lager	Dortmund	5+	Light hops, dry, estery
Bock	Lager	Bav., U.S. Can.	6	Full bodied
Doppelbock	Lager/ale	Bavaria	7-13	Full bod., estery, winey
Light beers	Lager	USA	4.2-5	Light bodied, light hops
<i>Top Fermented</i>				
Saisons	Ale	Belgium, France	5	Light, hoppy, estery
Trappiste	Ale	Bel. Dutch Abbeys	6-8	Full bodied, estery
Kölsch	Ale	Cologne	4.4	Light, estery, hoppy
Alt	Ale	Düsseldorf	4	Estery, bitter
Provisie	Ale	Belgium	6	Sweet, ale-like
Ales	Ale	UK, US, Can., Aus	2.5-5	Hoppy, estery, bitter
Strong/old ale	Ale	United Kingdom	6-8.4	Estery, heavy, hoppy
Barley wine	Ale/wine	United Kingdom	8-12	Rich, full, estery
Stout (Bitter)	Stout	Ireland	4-7	Dry, bitter
Stout (Mackeson)	Stout	United Kingdom	3.7-4	Sweet, mild, lact, sour
Porter	Stout	London, US, Can.	5.7-5	Very malty, rich
<i>Wheat-Malt Beers</i>				
(So. Ger.) Weizenbeer	Lager/ale	Bavaria	5-6	Full bodied, low hops
(Berliner) Weisse	Lager	Berlin	2.5-3	Light flavored
Gueuze-Lambic	Acid	Brussels	5+	Acidic
Hoegaards wit	Ale ale	E. of Brussels	5	Full bodied, bitter

Group 1 lists several famous lagers. These fully aged beers have distinctive flavor character and generally are named after the locality producing them. The next group consists of top fermented ales which have unique characteristics. The third group names several noted malted wheat beers.

Beer Constituents

The following brief review of beer constituents is intended to provide some basic information on beer that will enhance the readers comprehension of the sections on brewing materials and the brewing process which follow.

Table 3.2 lists the general types of compounds found in beer, gives their approximate concentration and their source(s).

Table 3.2 : Components of a "Typical" Beer

<i>Substance</i>	<i>Conc(%)</i>	<i>No. of Chem. Compds. Pres.</i>	<i>Source(s) or Agent</i>
Water	90	1	
Alcohol	4	1	Y, M
Carbohydrate	4	16	M, A
Inorganic salts	0.8	10	W, M
Nitrogen compounds	0.3	35	M
Organic acids	0.2	13	Y, M
CO ₂	0.5	1	Y
Other compounds	0.2	± 750	M, Y, H

^a Y yeast; M malt; H hops; A adjuncts; W wort

Water

Water is the major constituent of beer. Flavor character of the beer is influenced to some extent by the minerals present in the brewing water.

The presence of calcium and magnesium sulfates and carbonates assist the bitter, dry beer flavors brewed in Burton-on-Trent; lower levels of calcium sulfate and more bicarbonates permit the brewing of sweeter beers that are reduced in harshness. Such beers come from Munich and Dublin. The presence of both chloride and bicarbonate results in a beer that is sweeter in impression and more mellow.

Alcohol

Beer contains alcohol. Its concentration is generally from two to four two to four percent (grams per 100 grams) which in a properly brewed beer is the concentration of alcohol achieved when brewing grains are employed at their optimum concentration for flavor development.

Carbohydrates

The carbohydrates in beer are starch fragments left behind from incomplete enzyme degradation of the brewing grain starches. For the most part these fragments are glucose polymers of from four to about 24 glucose units. Regular beers contain from two percent to over four percent carbohydrate. The foam head and smoothness of flavor in beer is aided by this carbohydrate material. Table 3.3 gives a typical analysis of the carbohydrates found in a typical beer.

Table 3.3 : Carbohydrates in Regular Beers

	<i>Concentration Range (g/100mL.)</i>
Monosaccharides	0.1-0.2
Disaccharides	0.4-1.0
Trisaccharides	0.2-0.3
Polysaccharides (G ⁴ -G ²⁴) ^a	2.6-3.2

^a The G⁴-G²⁴ designates the size range of polysaccharides to be 4-24 glucose units.

Nitrogen Compounds

Aside from some ammonia and traces of purine and pyrimidine based material found in beer, the nitrogenous substances found in beer are primarily polypeptide in nature. The smaller polypeptides contribute a fullness aspect to beer as perceived in the mouth. They also add toughness to the foam bubbles resulting in a more stable and pleasing head of foam.

Inorganic Constituents

The inorganic components of beer influence its flavor and stability. The metallic taste frequently enhances the impression of harshness in beer. Iron and copper do this and the brewer exercises care to prevent exposure to these metals after the kettle boil.

Magnesium is also harmful to beer flavor. Iron, copper and magnesium all hurt beer stability.

Moderate levels of calcium benefit beer stability if present during fermentation. It will form insoluble salts with oxalic acid produced during fermentation which will precipitate, thereby removing this poorly soluble organic acid from beer. Calcium ion is essential to good brewer's yeast performance and flocculation.

Organic Acids

Lactic acid, acetic acid, 2-oxoglutaric acid, succinic acid , and other metabolic intermediate acids contribute to the pleasant tartness and bite that contributes to the flavor appeal of beer. These food acids do a flavoring job that is hard to duplicate with other acids such as the mineral acids.

Carbon dioxide

Carbon dioxide primarily enhances the flavor of beer. It adds to beer's acidic tartness; it pleasantly stimulates the pain sensors on the tongue and in the throat: its visible sparkle enhances the appearance of beer and the head of foam it displays.

Materials Used in Brewing

This section will list the brewing materials and to some extent will describe any preliminary treatment they will receive prior to entering the brewery. Examples of this are water treatment and barley malting.

A. Water

General requirements for a good brewing water will be touched upon here :

Water sources

Brewing water most generally comes from the public water supply. When it does not, it must come from an alternate supply or reservoir that is at least equivalent in quality to a public water supply. Sources of brewing water are either surface water taken from streams or impoundments or ground water made up of entrapped surface waters which have migrated through surface soil and rock structures to reside in underground natural voids known

as aquifers. The aquifers may be tapped by well bores and the water pumped to the surface or they may pass the ground water on to a lower level surface via a spring or seep.

Water purity

Brewing water, like any potable water, must be free from harmful substances. Public water supplies are monitored carefully for such potentially harmful substances as insecticides, heavy metal salts, detergents, herbicides, and various hydrocarbons. The number of substances that waters are screened for increases almost daily as we learn more about those pollutants that should be avoided. In recent years, considerable time and effort has gone into the reduction or elimination of polychlorinated biphenyls (PCBs), as well as chloroform and related compounds (halomethanes, chlorphenols, etc.) Those brewers who have their own water supplies must treat their waters just as the public utilities do.

Water Minerals

The influence on beer flavor character exerted by minerals is not totally due to the presence of specific ions. In some instances this is the case: the influence of chloride on sweetness, or calcium and sulfate on harshness, for example. Much, of the influence on beer flavor, however, is due to the interactions that these minerals have with other brewing ingredients such as grain and hop extracts during mash cooking and kettle boil.

Heavy metals

In India the Environmental Protection Agency has given several permissible contaminant levels for certain heavy metals and organic constituents in the potable water supply. Other countries have similar limitations. For the sake of example, these are given here:

Arsenic	0.05 mg/L
Barium	1.0
Cadmium	0.01
Chromium	0.05
Lead	0.05
Mercury	0.002 mg/L
Selenium	0.01
Silver	0.05
Nitrate	10.0 as N (44.0 as NO ₃)

Barley and Malt

The one grain that has been associated with beer since antiquity is barley. A member of the botanical family of grasses Gramineae the brewing barleys *Hordeum distichon* or *Hordeum vulgare*, are generally either two rowed or six rowed barley. A four rowed barley is favored in some parts of the world although its production is limited.

Barley

In two row barleys each node of the barley flower produces two grains of barley while six grains develop at each flower node of the six row barley.

Two row barley kernels are larger than the six row grains and much of the difference is due to the higher starch content. Germ size, protein content and husk content are more nearly equal on a per-kernel content. Thus the smaller six row kernels are higher in percentage content of these components.

(a) Harvest and storage

Barley is gathered quickly at harvest and is then stored until needed for malting, which might take a year. Its physical condition at the time of harvest and its storage environment are critical to malt quality and to the ultimate quality of the beer brewed from it.

(b) Weathering

If the grain ripening or the harvest season has been wet, weathering of the grain can occur in the field as bacteria and fungi naturally present on the grain surface find sufficient moisture to grow and infect the grain. The degree of weathering is critical to grain quality; the maltster and brewer avoid barley that shows any degree of weathering whenever possible.

Malt

Malting is basically the initiation of germination, carrying it to a desired end-point and then applying hot air to dry and stabilize the germinated grain so that the enzymatic activity that has developed will be preserved.

The final malt will have a moisture content of about four percent; it will tolerate several months of storage time and still

perform adequately in the brew house. The maltsters' skill must insure that the final malt has several properties:

(1) A specified level of alpha and beta amylase, (2) a specified level of solubilized protein including free amino acids and simple peptides, (3) a specified heat-generated color development in the water soluble malt components, (4) a minimal total count of microorganisms.

Fig. 3.1 consists of four photographs taken of a steep tank, a germination compartment and a kiln in a plant.

Fig. 3.1a shows the top of a steeping tank. The frothy surface of the steep water is the result of vigorous aeration: the cleaning effect this has on the grain is evident. The grain will initiate germination while in these tanks.

Fig. 3.1b shows the conical bottoms of the steep tanks and the valving that is necessary to provide water, aeration and grain removal for the steep tanks.

Fig. 3.1c shows a germination compartment filled with 13 kilo tons of barley. The grain has just been turned by the machine in the

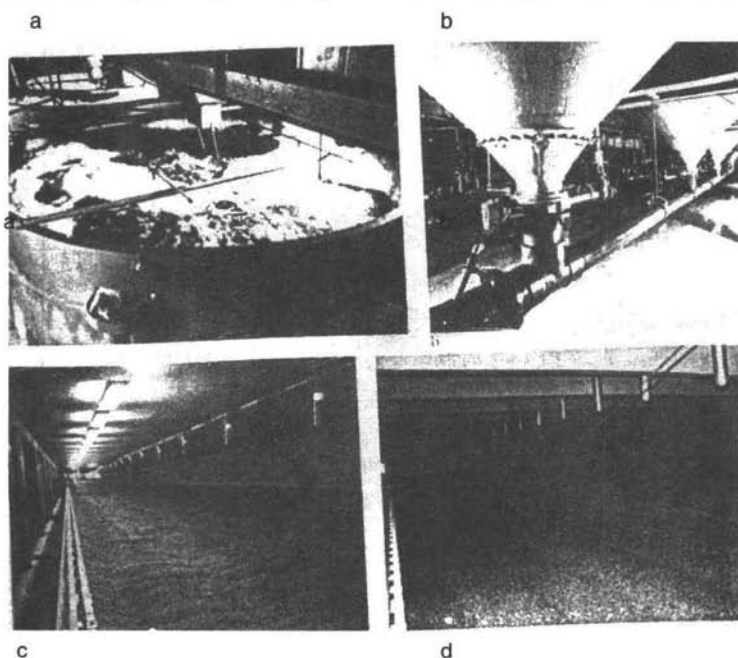


Fig. 3.1 : Pictures of malt plant equipment.

background. Air is being drawn through this grain bed from the bottom. Suspended over the center of the compartment is the sluicing loading system. It conveys the barley from the steep tanks to the germination compartment.

Fig. 3.1d shows a malt kiln containing over 170 kilo tons of malt that is being dried with warm air.

Brewing Adjuncts

Although adjuncts are used mainly because they provide extract at a lower cost than that available from malt, other definite advantages are achieved.....beers of lighter color with a less satiating, snappier taste, greater brilliancy, enhanced physical stability and superior chill proof qualities. These are the attributes that have been found essential to a product that must survive the shipping and storage conditions that exist throughout these continents.

Table 3.4 lists the various adjunct materials that are used in the brewing of beer.

Table 3.4 : Adjuncts Used in Brewing

<i>Cereal Source</i>	<i>Form of Product</i>
Corn	Dry milled grits-germ and skin free Pregelatinized grits or flakes Refined grits (starch)
Rice	Rice grits-broken grains flakes Pregelatinized rice grits
Corn syrup	Can add at end of kettle boil
Corn sugar	Glucose from starch conversion
Sorghum grits	From kaffir corn and milo maize
Barley	Dehusked, milled whole grain
Wheat	Low protein wheat flakes

Hops

The hops grown today do not differ significantly from the early hop strains employed. Culturing practices have refined the growing of these plants; hardiness and disease resistance are sought in the hop varieties that will become root stocks but which will receive vine transplants of other hop varieties having desired flavor and storage properties.

Hop utilization

Only the dried cones of the female hop plant are collected for brewing. They are kiln-dried and baled; shipping and storage are done under refrigeration to retard oxidation of the hops.

The small lupulin glands contain both the bittering resins and the essential oils that are sought by the brewers. The bracteoles contribute little except polyphenolic material which enhances the astringency and tea-like character found in beers.

Hop chemistry

Table 3.5 lists the substances in hops that can influence the flavor of beer. The alpha and beta resin acids are the most desirable hop constituents. Humulone is generally chosen as the representative alpha acid although cohumulone and adhumulone are essentially equivalent. The kettle boil isomerizes most of these and it is the refreshing sharp clean bitterness of isohumulone, isocohumulone and isoadhumulone that adds much to the flavor appeal of beer.

Table 3.5 : Substances in Commercial Hops that Influence Beer Flavor

<i>Substance</i>	<i>Concentration Range (%) in Hops</i>	<i>Flavor Effect in Beer</i>
Bitter resins:	6-17	
Alpha acids		
humulone		
cohumulone	5-8	Bitter
adhumulone		
Beta acids		
lupulone		
colupulone	7-10	Bitter
adlupulone		
Hydrocarbons:	0.2-0.6	
myrcene	0.1-0.3	
pinenes	0.003-0.005	
caryophyllene	0.05-0.08	Aromatic
farnescene	0.07-0.1	Terpene-like
humulene	.08-0.15	
selinenes	0.005-0.015	
Oxygenated esters	0.05-0.1	Floral
Tannins	3-5	Harsh, astring.
Lipids and wax	2-4	Dulling

Schematized below are the changes which occur when humulone is isomerized to isohumulone (Fig. 3.2). The beta acids in fresh hops do not contribute greatly to beer flavor; they are not sufficiently soluble. In old hops where they have had time to undergo oxidative change to the more soluble hulupones, they do exert a flavor influence although not so pleasing to the palate as the isohumulones.

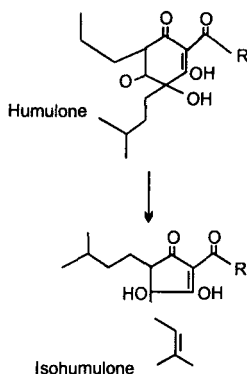


Fig. 3.2 : The heat conversion of humulone and isohumulone.

The hydrocarbon-like essential oils are powerful aroma producers and can exert a flavor influence at low (0.3-0.5 ppm) concentrations. At higher levels they overpower other aromas and are too terpene-like to be pleasing. The kettle boil is useful in evaporating or oxidizing these materials leaving the desired residual concentration.

Other oxygenated terpenes are alcohols, aldehydes, acids, and esters of the hydrocarbon terpenes and terpene fragments. Because they and their oxidation products are more pleasing than the hydrocarbon terpenes, they are desirable hop components for beer.

Polyphenols are extracted from hops during the kettle boil. Some of these have molecules of gallic acid chemically bound to a molecule of glucose (gallotannins); others are polymers of various phenolic compounds related to catechin.

Hops are marketed to the brewer as baled dried hop cones, pellets made from hop cones or extracts prepared from hops. Hops are added into the brewing process at the kettle boil although there is some practice of "dry hopping" which is the addition of hops to

the beer while it is in the aging cellar. Purified pre-isomerized isohumulone preparations are also available for direct addition to the finished beer.

Brewer's Yeast

The primary function of brewer's yeast is to convert the fermentable sugars present in wort to ethyl alcohol and carbon dioxide. It also propagates itself and releases many substances incidental to its metabolism that significantly influence beer flavor.

The Brewing Process

Brewhouse Operations

In the following discussion of the brewing process, an effort will be made to describe the various steps from two perspectives. One will be from a practical brewing standpoint where production and engineering aspects will be touched upon; the other will be from a chemical and biological standpoint describing some of the chemical changes that occur and identifying the agent responsible.

1. Milling

The brewing process is initiated by milling the malt and, where used, the adjunct grain. This operation is designed to reduce the starchy grain to a standard particle size while doing no more damage to the grain husk than is necessary.

a) Malt milling

Three complex objectives of malt milling are:

1. To split the husk mostly lengthwise to expose the endosperm.
2. To crush the endosperm making all its constituents accessible to enzyme activity.
3. To keep the percent of fine particles to a minimum, thereby minimizing excessive dough formation in the mash.

This milling activity is in reality a crushing of the grain between two or more rolls which are set to a desired separation and tension for crushing the malt. The individual speed of the rolls may be varied to introduce some shearing friction to the grain to supplement the crushing action of the rolls. Modern mills are multi-roll in design, containing five or six rolls.

b) Wet milling

In recent years wet milling has been employed. Here the whole uncrushed malt is soaked in hot water until the kernel loses much of its brittleness and contains up to 30% moisture. The wet malt is then crushed between rolls where the endosperm material is squeezed out of the husk which remains more intact providing a better filter medium for clarification of the wort after mashing.

c) Adjunct milling

The method of milling called for here depends largely on the material. Most adjunct material has gone through preliminary processing of some kind and requires little or no additional particle size reduction. Some corn (maize) grits, corn starch and other cereal starches are in this category. Rice, rice grits and large cereal grits which must be milled are generally reduced to the smallest particle size possible. They are free of husk and the germ material, if present, poses no threat to good wort filtration.

2. Mashing

Two basic approaches to mashing are employed. The infusion mashing system uses a single mashing temperature (65°C) and is highly suitable for the well modified malts. Mashing is usually done in a single vessel. The other, the decoction mashing system, it employs a multi-temperature system and, most of the time, interfaces with a cereal cooker used for the adjuncts.

3. Lautering

a) The lauter tub

When mashing is completed the total hot mash is transferred to a lauter tub. This false bottomed straining device separates the liquid wort from the insoluble grain residues. A lautering machine is in the lauter tub; it consists of two or three booms suspended horizontally from a central rotating axle. The booms have vertically suspended blades or rakes that can be manipulated to constantly keep the grain bed loose as the booms rotate around the lauter tub.

The insoluble residues are levelled off in the freshly filled lauter tub and, after a brief settling period, the wort is allowed to flow down through the grain layer where the grain residue will assist in retaining small insoluble grain particles while allowing the clear

wort to diffuse downward and through small straining slots in the false bottom of the lauter tub. The rotating arms and movable blades operate just enough to keep the grain bed from plugging or blinding which would prevent flow of the clear wort.

b) Run off

As the wort flows from the lauter tub it enters a vessel called a grant which serves as a surge vessel by retaining the wort briefly. This permits the brewer to inspect the wort and return it to the lauter tub until the grain residue layer properly clarifies it. At this juncture the brewer will manipulate valving at the grant and send the clear wort on to the kettle.

c) Sparging

Sparge water is added to the top of the grain bed before it has a chance to drain dry. This sparge water dilutes the wort lowering its viscosity which enhances its flow through the grain bed. It also diffuses away the wort that is dissolved into grain particles and malt husk and carries it on into the main wort stream. This sparge water is used carefully: it dilutes the brewing wort and this dilution must be carefully controlled so that maximum wort recovery is achieved. Since the sparge water ends up in the wort, it naturally must be of brewing quality.

d) Wort filtration

Several brewers today employ large plate and frame filter presses to filter their wort rather than lauter it. In principle they strain and sparge the grain much as is done in lautering, except that the drainage distances are shorter.

e) Spent grain removal

After the wort has been adequately removed from the spent grains and sent to the kettle for boiling, the grain residue is dropped through grain valve openings in the floor of the lauter tub. The lauter machine arms and blades assist in moving the spent grains to these valves. The grain mass ends up in a holding tank from which it will be dropped wet into grain hauling trucks or it will be sent to a grain dryer located nearby. Whether sold wet or dry these spent grains are used as animal feed supplements. Since the grain starch has been

removed, the spent grains are relatively high ($\pm 30\%$) in protein content.

4. Wort Boiling

Brew kettles vary greatly in size; small volume kettles holding less than a hundred hectoliters are still in use although large modern kettles approach ten times this volume.

a) Heating

Heat for the kettle can be provided by direct firing although most heat is provided by steam applied indirectly.

b) Functions of wort boiling

Purpose of the kettle is to boil the wort under conditions that permit slight oxidation to occur. The major desired effect of wort boiling is stabilization of the wort.

Certain heat coagulable polypeptides must be coagulated by the kettle boil. If they are not, they will slowly denature and/or react with phenolic material to produce insoluble hazes in the finished beer package. The kettle boil serves functions other than stabilization. Hops are added to the wort during boiling.

The kettle boil also brings about some wort concentration, generally about 10% giving a slightly higher level of solids in the final fermentation wort. A rolling boil is desired and the kettles are designed to bring this about. The swirling pattern of the bubbles of steam generated is essential to the development of large flocculant particles of the complexed insoluble material. This insures their easy removal. The rolling boil aids evaporation, maintains a uniform temperature with minimal overheating which causes excessive color development and dangerous boiling over. Distillation of the unwanted volatile substance is also enhanced by the rolling boil.

c) Hop extraction and conversion

When dried hops are used (or hop "kettle" extract) they are added to the boiling kettle on a schedule.

Some of the hop resins and phenolic substances are lost in the kettle. They enter into the complexing reactions with polyvalent metal ions, primarily iron and copper. This complex insoluble material removes the iron and copper from the wort sufficiently to avoid resultant flavor problems in the beer.

5. Wort Cooling/Trub Removal

a) Hot trub

The insoluble flocculent material formed during kettle boil is called hot trub; somewhat similar material is present that is soluble at hot temperatures but precipitates when the wort is cooled. Not surprisingly, this is called cold trub (cold break). Both trubs should be removed.

6. Brewhouse and Cellar Sanitation

Brewhouse equipment up to the refrigerated wort cooler is cleaned by standard cleaning procedures which have little or no involvement with sanitation precautions. Beginning with the refrigerated wort cooler, and continuing throughout fermentation, cleaning practices must meet stringent requirements, both for general cleanliness and for complete sanitation.

a) General practices

Both the frequency and the intensity of cleaning are stepped up considerably. Brewers have long known that stringent cleaning is the best sanitation practice they can employ; good cleaning is followed by the use of acid or caustic to achieve bactericidal conditions. The application of heat or the use of germicidal agents is employed.

Wort Constituents

A brief review of brewing wort constituents will be given here. They are significant to what follows in brewing, both to yeast nutrition and to those compounds that persist on into the beer. Table 3.6 lists the wort compounds and gives their concentrations in an "average" wort.

Table 3.6 : Wort Constituents and Their Concentration

<i>Substance</i>	<i>Concentration (%)</i>
Total carbohydrates	90-92
Nitrogen compounds	3-6
Salts and minerals	1.5-2
Acids	0.5-1
Phenols/polyphenols	0.1-0.2
Hop resins, oils and esters	0.05-0.1
Lipids	0.03-0.06

1. Carbohydrates

A typical brewing wort contains about 11% soluble grain extract; about 90% of which is carbohydrate. This carbohydrate fraction has been found to contain pentoses (0.03%) , hexoses (9%), disaccharides (52%), trisaccharides (13%), and polysaccharides (26%). The pentoses are non-fermentable. The hexoses and disaccharides are the tetrasaccharides and larger polysaccharides (alpha glucans) are non-fermentable and contribute an aspect of fullness to beer and denseness to its head of foam.

One minor component of the polysaccharides in wort, the beta glucans are normally present at 200-300 parts per million and at that concentration are no problem for the brewer.

2. Nitrogenous compounds

Virtually all of the nitrogenous compounds in wort come from malt. Consequently, worts made from all malt will have relatively high levels (over 1000 mg/liter) of wort nitrogen; average North American worts, because of adjunct usage, are lower (600-800 mg/liter).

About 30% of this wort nitrogen is in the form of free amino acids; another 30% is made up of peptide chains containing from 2 to 30 amino acids. Wort polypeptides larger than 30 amino acids have marginal solubility and efforts must be made to eliminate any survivors that might still persist in the final beer. A notable exception, however , is a neutral protein fraction having 100-150 amino acids which is quite soluble and complexes with carbohydrates and hop resins to form the foaming agent in beer.

About 20% of wort nitrogen is made up of purine and pyrimidine based compounds. Yeasts can utilize the free bases that are present.

Traces of ammonia, volatile amines and amino alcohols are found in wort. They are of little significance.

3. Inorganic Constituents

Inorganic ions are present in wort at levels between 1.5-2%. Calcium, sodium, chloride and sulfate ions are primarily from the brewing water. Both water and malt are responsible for magnesium, manganese, copper, iron, and zinc. These ions are all required as

cofactors for various enzymes. Potassium and phosphate come primarily from malt.

4. Vitamins

Thiamin, riboflavin, niacin, pantothenate, biotin, pyridoxin, mesoinositol and p-aminobenzoate are all present in wort at levels above the brewer's yeast requirements; thiamin is most depleted by the yeast. With the exception of niacin, total levels of the *B*-vitamins in modern beer perhaps should not be considered to contribute greatly to human nutrition. With the exception of niacin and perhaps biotin, the levels in filtered beer are only fractions of man's minimal daily requirement. Table 3.7 shows the levels of some vitamins in a typical American brewing wort and in the beer brewed from it.

Table 3.7 : Some Vitamins Found in a Wort and in a Beer Brewed From It

	Wort (mg/L)	Beer
Thiamin, HCl	0.3	0.02
Riboflavin	0.18	0.18
Niacin	2.4	0.6
Pyridoxin	0.2	0.02
Pantothenic acid	0.25	0.4
Biotin	0.01	0.003
Inositol	0.04	0.04

5. Polyphenols

Hops contribute some hydrolyzable (gallic acid based) polyphenols which are fragments of the gallotannins in hops. Malt is the source of most of the non-hydrolyzable or condensed tannins. These vary from simple non-polymerized phenols to more complex polyphenols based on hydroxybenzoic acid, hydroxy-cinnamic acids, flavinols, cyanins and catechins. They are extracted from the malt husk and the outer layer (aleurone) of the grain itself.

6. Hop compounds

Those compounds of most significance to brewing will be dealt with again here. The desired bittering substances are the isomerized

alpha acids isohumulone, isocohumulone and isoadhumulone. They are complex isoprenoid structures which differ in a fatty acid side chain attached and were formed from their non-isomerized counterparts during the kettle boil.

Some polyphenolic materials are extracted from hops and contribute tanninlike astringency to beer flavor. They also cause problems to beer stability due to their tendency to either degrade or polymerize upon oxidation.

Today there is significant use of various hop extracts by brewers throughout the world. These vary from simple vacuum concentrated alcoholic extracts to highly purified pre-isomerized alpha acid preparations.

7. Melanoidins and Phenolic Pigments

These pigmented compounds are present in beer at fractional percent levels. Pigmented products of browning reactions are the major color producers in golden amber colored beers. Darker brown beers have more intensive browning reaction products present but they have caramel reaction products also.

The darker colors are formed by applying more intense heat to the malt to achieve caramelization and, in the case of black malt, actual charring of the malt occurs. Some dark beers may be colored by an acid stable caramel coloring.

Beer oxidation colors develop as beer ages: these generally are the result of oxidative condensation of the beer phenols. This coloration has a foxy red hue and generally is accompanied by a haze development as some of the polyphenolic condensation products become too large to remain in solution. The resultant haze frequently will cause canned or bottled beer to gush when opened. Both the foxy red color and gushing tendency are characteristic of old packaged beer.

8. Lipids

Although present in beer at levels generally below 50 ppm, the lipid fraction can be a significant factor in beer quality. Some of the simpler unsaturated fatty acids in wort are esterified to ethyl and butyl esters, which cause pleasant aromas. The poly-unsaturated fatty acids, primarily linolenic and linoleic acid, tend to limit the shelf life quality of beer. The fatty aldehydes which are the initial

oxidation products of these fatty acids are responsible for the rancid fat, cardboard-like flavor best exemplified by *trans*-2-nonenal which is detectable in beer at 0.1 parts per billion and is generally unpleasant at about 3 ppb.

Cellar Operations

Once wort is chilled, the cold temperatures are maintained from that point on until the final packaged beer is pasteurized; non-pasteurized draught beer is kept cold until consumed.

This section is captioned "cellar operations" because classically the operations described are carried out in cold areas called brewing cellars.

Several brewing functions are carried out in the cellars. These include wort aeration, yeast pitching, primary fermentation, yeast recovery and reuse, pure culture systems, aging and finishing.

Yeast recovery and reuse

The settled yeast is removed from the fermented wort in different ways. Some brewers decant the supernatant young beer and send it to aging tanks, others withdraw the yeast leaving the young beer undisturbed.

Washing and preparation

Yeast recovery systems vary in design of equipment but the basic procedures employed are quite similar. The settled yeast in the fermenter is pumped to the yeast washing and storage area where it is washed free of trub and other wort sediment. It is then held cold ($\pm 2^{\circ}\text{C}$) until it is needed to pitch another fermentation. Many brewers reuse their yeast only a few times; others continue to reuse the yeast indefinitely. Those having longer usage generally practice more stringent anti-bacterial measures during yeast washing. They add phosphoric acid or tartaric acid to obtain a pH of 2.2 and hold the yeast for about two hours before removing the yeast.

Yeast disposal

Yeast that is surplus from primary fermentation and from the aging cellars has marked value. It can be dried "as is" and sold as a feed supplement or it can be washed free of occluded bitter hop resin material, dried and sold into the human food and

pharmaceutical market. It can also be subjected to some form of plasmolysis that releases soluble cell content. This material can then be separated from cell debris and concentrated to a thick paste or dried. It enjoys usage as a food flavorant and in some areas is consumed directly as a spread.

Table 3.8 gives the concentration of some of the B complex vitamins in dried brewer's yeast.

Yeast Pure Culture Systems

Brewers who recycle their brewer's yeast a minimal number of times employ a pure culture system to keep a quantity of fresh yeast coming into the plant.

**Table 3.8 : Vitamin Content of Dried Brewer's Yeast
(REED and PEPPLER, 1973)**

Vitamin	<i>S. uvarum</i> ($\mu\text{g/g}$)	<i>S. cerevisiae</i> ($\mu\text{g/g}$)
Thiamin	9-40	9-40
Riboflavin	45	36-42
Niacin	400	320-1000
Pyridoxine	40	25-100
Folid acid	5	15-80
Pantothenate	100	100
Biotin	1	0.5-1.8

Culture propagation

The pure culture system must start with the establishment of stock cultures. A single cell isolate is prepared from an initial culture and is grown on an acceptable laboratory medium. Yeast cells harvested from this culture may be inoculated into working stock media. These can be held at 2-4°C for several months before subculturing must be done.

The pure culture system is started by inoculating a small flask of sterile aerated wort with a few milligrams of yeast from a stock culture tube. This flask culture will grow and be ready to transfer *in toto* to a larger flask culture in about four days. The liquid volume in the second flask (aerated wort also) should be about ten times the volume in the first. When this flask culture has grown, it will generally be moved from the laboratory to the pure culture system.

There a small tank of about ten times the large flask volume receives the contents of the flask. Standard practice is to continue this tenfold stepwise scale-up until sufficient yeast has been grown to pitch a commercial-scale fermentation.

Laboratory checks

The each step of the scale-up, the developing yeast population is examined carefully. The yeast is screened for evidence of any aberrant metabolic behavior and the culture is screened for contaminants. All transfers are made under stringently aseptic conditions.

Aging and Finishing

As the fermenting stage comes to an end and the fermenter beer is separated from the settled brewer's yeast, the young beer can either be subjected to aging (lagering) or it can be finished straight away. If no further aging is planned, the primary fermentation will be allowed to reach the final desired attenuation (sugar fermentation) before cooling is applied to cool the fermenter and drop out the brewer's yeast. Thee flocculent nature of *S. uvarum* is helpful at this juncture.

If further aging is desired, the young beer (as noted above) is moved to an aging (lager or storage) tank where the beer will be subjected to gradually lowered temperatures as the yeast completes fermentation.

Author lists five major process functions that must be carried out in aging and finishing. These are:

- (a) Flavor maturation
- (b) Carbonation
- (c) Standardization
- (d) Chill proofing and stabilizing
- (e) Clarification

Microbial Contaminants in Beer

Beer spoilage microorganisms, both bacteria and yeast, are widespread in brewing; however, most of the time their numbers are too low to be significant. Well operated breweries are not necessarily run aseptically but they do exercise the microbiological control needed to keep the spoilage organisms from propagating.

Bacterial Contaminants

Beer contains alcohol and hop materials and has a low pH; it contains no oxygen and very little fermentable sugar (generally). These features severely limit the variety of bacteria that can grow in it.

Gram Positive Bacteria

Members of the genus *Bacillus* are aerobic sporeforming bacteria which can develop quickly and several strains are thermophilic. They only threaten sweet worts that are held too long as in delayed extraction of the wort from the spent grains during lautering.

Lactobacillus

Members of the genus *Lactobacillus* make up the predominant spoilage organisms for beer. There are several species of lactobacilli that grow in beer. Homofermentative *L. delbrueckii* produces essentially only lactic acid from glucose. It is a thermophile that can grow at sweet wort temperatures. *L. casei* and *L. plantarum* are not heat resistant but are homofermentative and are found only in the brewhouse. Several heterofermentative lactobacilli which convert glucose to lactic acid, CO₂, acetic acid and ethanol are now called *L. brevis*. An approved list of bacterial names has designated *Lactobacillus brevis* as the official name for several lactobacilli. These include *Saccharobacillus pastorianus*, *Lactobacillus pastorianus*, *L. pastorianus*, var. *brownii*, *L. pastorianus* var. *lindneri*, *L. pastorianus* var. *diastaticus*. Another lactic rod, *L. buchneri*, now is the approved name for *L. frigidus* and *L. parvus* (PRIEST, 1981). Several of the beer spoilage lactobacilli have been specifically identified using immunochemical techniques.

Off-flavors produced by these heterofermentative lactic rods vary. Some strains will do little other than lower the beer pH slightly; others (most lactobacilli) will produce troublesome levels of diacetyl. They all produce a silky, flowing, almost pearlescent type of turbidity that is quite characteristic.

Diagnostic culturing of the lactobacilli can be difficult for some strains. Many of these organisms appear to have some impairment of their ability to break down disaccharides; enriched media help in culturing them.

Pediococcus

Somehow it seems poetic justice that restore the name *Pediococcus damnosus* to *Pediococcus cerevisiae*. That best describes what this organism does to beer. Workers reports that over 97% of 840 isolated brewery cocci were found to be *P.damnus*. The others were *P.inopinatus* and *P. pentosaceus*; neither strain produced diacetyl and spoiled beer as did *P. damnus*.

The pediococci form characteristic tetrads when growing well; they always exhibit numerous diplococcal forms. They vary in their sensitivity to oxygen and in their requirement for CO₂ tension; they can be satisfactorily cultured on enriched media.

Miscellaneous cocci

Other micrococci or staphylococci will be found in beer; they do not grow there but can survive in beer for long periods of time. They apparently cause no problems. Microorganisms isolated and identified from beer are *Micrococcus kristinae*, *M. varians*. *Staphylococcus epidermidis* and *S. saprophyticus*.

Gram negative bacteria

The gram negative organisms associated with brewing have had some name changes recently.

Acetic acid bacteria

The acetic acid bacteria have been divided into two groups, *Acetobacter* and *Gluconobacter*. The *Acetobacter* will oxidize ethanol to CO₂ and water via the tricarboxylic acid pathway; if a strain has flagellae they will be peritrichous. The *Gluconobacter* will only oxidize ethanol to acetic acid via the hexose monophosphate pathway; if flagellated, the position will be polar.

The organisms are obligate aerobes, thus they are only spoilers of beer that is exposed to the atmosphere. Good packaging and good housekeeping keep them in check.

Identified species include *Acetobacter aceti* subsp. *xylinum*, *A. pasteurianus* subsp. *pasteurianus*, *Gluconobacter oxydans* subsp. *oxydans* and *G.oxydans* subsp. *industrius*.

Zymomonas

Zymomonas mobilis or *Z. anaerobia*, as it was formerly called, is readily killed by a few minutes exposure to 60°C. It therefore can only be a threat to draught beer or ale, especially when priming has been done with sucrose or glucose. This organism produces ethanol from glucose or fructose via the Entner-Doudoroff pathway; there is some leakage of acetaldehyde. Both acetaldehyde and H₂S are released to the beer or ale. Cleanliness of equipment will help avoid contamination. Rigid sanitary measures are called for to clear up an infection.

Enterobacteriaceae

This family of bacteria has been regrouped considerably. Some lists the genera that are significant to brewing. The following list gives these organisms and their former names;

Approved name	Synonyms
<i>Citrobacter freundii</i>	<i>Escherichia freundii</i>
<i>Enterobacter aerogenes</i>	<i>Aerobacter aerogenes</i>
<i>Enterobacter agglomerans</i>	<i>Erwinia herbicola</i>
<i>Enterobacter cloacae</i>	
<i>Hafnia alvei</i>	<i>Enterobacter alvei</i>
	<i>Enterobacter hafniae</i>
<i>Klebsiella pneumoniae</i>	<i>Aerobacter aerogenes</i>
<i>Obesumbacterium proteus</i>	<i>Flavobacterium proteus</i>
	<i>Hafnia protea</i>

Obesumbacterium proteus is a facultative anaerobe that can grow well with brewer's yeast. It is a short, fat rod that, when present and growing, is responsible for an odor resembling parsnips. Its metabolic pathways are similar to those in brewer's yeast; it is a facultative anaerobe. This organism grows well until the brewer's yeast shifts the pH of the wort to 4.5 or below. Fresh wort should

be pitched quickly to minimize growth of these organisms and yeasts cultures should not be used if they are present.

Miscellaneous wort organisms

Several genera of microorganisms can be isolated from wort. They are non-fermentative and only grow until all oxygen is gone. Many of them can survive under brewing conditions and will enjoy another brief growth spurt when repitched along with the yeast. This can be avoided, of course, by acid washing the yeast with phosphoric acid or ammonium persulfate.

Table 3.9 lists the more common brewery spoilage organisms and that stage of production they infect.

Wild Yeast Contaminants

Perhaps the best definition of wild yeasts is that they are unwanted yeasts that threaten quality of the beer.

Table 3.9 : Brewery Spoilage organisms and the stage of Production they Infect

<i>Production stage</i>	<i>Organisms</i>
1. Mashing/sweet wort	Thermophilic lactics
2. Cool wort to pitching	<i>Obesumbacterium</i> <i>Lactobacillus</i> <i>Acetobacter</i> <i>Gluconobacter</i> <i>Enterobacteriaceae</i>
3. Fermentation	<i>Obesumbacterium</i> <i>Acetobacter</i> <i>Gluconobacter</i> <i>Enterobacteriaceae</i> <i>Lactobacillus</i> <i>Pediococcus</i>
4. Package in trace	<i>Acetobacter</i> <i>Gluconobacter</i> <i>Zymomonas</i> <i>Lactobacillus</i> <i>Pediococcus</i>

Beer Spoiling Yeasts

Many such yeasts are strains of *S. cerevisiae* that can cause off-flavors, ferment dextrins and cause turbidity in beer.

Table 3.10 lists the common species involved in beer spoilage.

Packaging Operations

Beer in much of the world is primarily consumed from a package.

The brewer employs three basic packages to get his beer into the hands of the consumer; bottles, cans and kegs.

Table 3.10 : Some Wild Yeast Reported to Spoil Beer

<i>Bretanomyces</i>	<i>bruxellensis</i>
	<i>lambicus</i>
	<i>anomolus</i>
	<i>clausenii</i>
	<i>intermedius</i>
<i>Candida</i>	<i>utilis</i>
	<i>lambica</i>
<i>Debaryomyces</i>	spp.
<i>Dekkera</i>	<i>bruxellensis</i>
	<i>intermedia</i>
<i>Hanseniaspora</i>	spp
<i>Hansenula</i>	<i>anomala</i>
<i>Kloeckera</i>	<i>apiculata</i>
<i>Pichia</i>	<i>membranaefaciens</i>
<i>Rhodotorula</i>	spp.
<i>Saccharomyces</i>	<i>cerevisiae</i> var. <i>ellipsoideus</i>
	var. <i>turbidaus</i>
	<i>bayanus</i> (<i>pastorianus</i>)
	<i>diastaticus</i>
	<i>inuitatus</i>
<i>Torulopsis</i>	<i>colliculosa</i>
	<i>inconspicua</i>

Bottling Operations

Filtered beer is sent through a sterile, CO₂ purged release line into the packaging area; there it is stored in a tank until needed at the bottle filler. The beer is checked for air pick-up during transfer and for clarity. Most other tests of the beer are done at the filtered beer tank in the brewing operation. Quality control (Q.C.) checks on functions of the beer transfer system.

Filling

This beer is sent to a filler bowl which acts as a surge tank during filling. Most fillers today are rotary fillers which can fill up

to 1400 bottles per minute with quiet fills that excite the beer very little. Properly done, virtually no air is introduced into the package.

Pasteurization

The filled bottles are pasteurized in tunnel pasteurizers. These pasteurizers have zones which are heated to varying temperatures which will heat the bottles as quickly as possible without causing temperature shock breakage. The bottles are heated to slightly above 60°C and are then cooled down as quickly as can be done without temperature shock breakage. Pasteurization target is to obtain a time-temperature dosage that will equal that which is obtained when the coldest spot in the bottle is held for from about 6 to about 15 minutes at 60°C. These are called 6 to 15 pasteurization units (P.U.) One P.U. is developed when beer is held at 60°C for 1 minute. 6 to 15 P.U.s is adequate pasteurization for almost all packaging situations. If the beer is heavily infected or if highly heat resistant organisms are present in significant numbers, then this P.U. dosage may need to be increased.

Light Struck Beer

Bottled beer has a problem with light exposure. All beer that has been brewed using natural hops in the normal brewing procedure develops sunstruck flavor when exposed to visible light. The photolytic cleavage of the hop bittering substance, isohumulone, releases an isoprene diene that reacts with the traces of hydrogen sulfide present in beer to produce a mercaptan that is quite potent in flavor.

The mercaptan is 3-methyl-2-butene-1-thiol; it has a skunky odor that is not pleasant in beer. To avoid its development, beer must be protected from direct light. Beer bottles must be placed in paper cartons which shield them from light. Dark amber bottles help retard this action by light. If hop extracts are employed in brewing, they can be chemically reduced to *p*-isohumulones which are able to resist the photochemical splitting off of the isoprene radical.

Canned Beer

Today beer cans are essentially all two piece (one piece can body+lid) cans made of either aluminum or tinplate. They are coated on the inside with epoxy which covers the metal well, leaving essentially none exposed to the beer.

Can filling

Freshly rinsed cans are filled with beer on rotary fillers at speeds up to 2000 cans filled per minute. These high speed units can be finely tuned to operate at high filling speeds while introducing negligible air (oxygen) into the beer.

Pasteurization

Can pasteurization is done similarly to bottle pasteurization. Here the speed through the pasteurizer can be faster; the can is not susceptible to temperature shock breakage and the can metal will transfer heat (or cooling) more readily than will glass. The filled cans are packed into trays and are ready for shipment.

Shelf Life of Packaged Beer

Both cans and bottles have two enemies that can do harm to the beer they contain. One is oxygen, the other is temperature.

Oxygen

If oxygen gets into the beer at any juncture after the brewer's yeast has been removed, shelf life expectancy of the beer will suffer. Pasteurization will force oxidation to occur, reacting about 80% of the oxygen that has been introduced to the beer. No brewer wants his product going into the market place with more than 1 ppm of oxygen present. The simple phenols and polyphenolic materials present will undergo flavor change as well as complex with other polyphenols and peptides present to form hazes. Other oxidation reactions will result in the appearance of furfural and 2-*trans-nonenal*, both of which are "old beer" flavors and do not connote freshness. The beer will alter in color, changing from a characteristic yellow-amber to take on a foxy red hue. Hop bitterness is no longer clean and fresh; it is duller, tends to linger on the back of the tongue, and is no longer as refreshing as it is in fresh beer.

The condensed polyphenols precipitate forming a light haze made up primarily of light flake-like particles. In American beers, where carbonation frequently is over 2.7 volumes of CO₂ per volume of beer, these particles will act as nuclei for CO₂ bubbles. The tendency to do this is at the time of pressure reduction from opening and the result is rapid CO₂ bubble evolution from the bottle. Entrained beer becomes foam as it surges out of the package opening and fountains into the air.

Many of these changes occur in beer as a normal result of shelf life aging. It may take several months for this to occur, however, and the brewer tries to keep his shelf life duration considerable shorter. With oxygen in the bottles, these changes can all occur in 2-3 weeks.

Temperature

When perfectly packaged, beer is at its flavorful best the day it is placed in the bottle or can. Undesirable oxidation reactions begin at that juncture and continue throughout shelf life of the product. Temperature of the package plays an important role in aging, the oxidation reactions for the most part are simple first-order reactions and the cooler the product can be held the slower the oxidation reactions will be. A good illustration of this is the observation that beer held for the one week at 38°C is roughly equal in freshness to beer held at 29°C for one month or at 5°C for over a year.

Draft Beer

The freshest beer that a consumer can drink is draft beer. It is not pasteurized and it is kept cold until in the hands of the consumer.

Cooperage

The widespread use of stainless steel cooperage today has been responsible for considerable improvement in draft beer quality. The relatively smooth, non-porous surface presented by stainless steel is not only readily cleanable; it is sufficiently inert chemically to allow the use of strong alkaline cleaners and high temperatures. These, of course, result in both excellently cleaned and essentially sterile cooperage. Many brewers still use aluminum cooperage and have few problems. Aluminum is more reactive towards beer than is stainless steel so the aluminum kegs are coated with a thin layer of paraffin or microcrystalline wax. The newer tapping devices keep the cooperage sealed even after it has been emptied and removed from the dispenser.

Quality Assurance

Physical and Chemical Measurements

The quality assurance functions in most instances are custodial. Brewery management sets the specifications for the

product; the brewmaster establishes the brewing process, equipment requirements, raw material requirements, etc. and somewhere in management, the flavor requirements are established. Quality assurance develops, or is given, the analytical procedures that must be done to insure that the brewing process is "on target" and the finished product has the required integrity. The function of quality assurance is to measure and report on (ranked chronologically, not in importance):

1. Adequacy of cleaning and sanitation (plant and equipment).
2. Acceptability of raw materials.
3. Biological status of beer in process.
4. Chemical and physical analysis of the finished beer.
5. Flavor analysis of the packaged beer.

Cleaning and Sanitation

Housekeeping and sanitation must be maintained properly if cellar activities and packaging activities are to be kept free from troublesome microbial infections. The combination of pH, alcohol content and CO₂ content limits the beer infectors to non-pathogenic microorganisms. So the threat of these microorganisms is beer spoilage, not human health.

Raw Materials Acceptability

Responsibility for raw materials quality is a shared one. The brewmaster generally places specific requirements that these materials must meet; those that can be measured chemically or physically are turned over to Quality Assurance. He may also share some of the flavor and "hand" evaluation chores with Q.A. Table 3.11 lists several of the evaluations that are made of various brewing materials.

Biological Survey of Beer "in process"

The brewmaster is given interim data describing progress of each fermentation and/or aging tank in his cellar system. This information is provided in part by his own production people and in part by the Q.A. people. The information obtained by laboratory analysis is provided by Q.A. Table 3.12 lists those measurements that are done in the lab.

Table 3.11 : Raw Materials Analytical Procedures Done by Quality Assurance

<i>Malt</i>	<i>Hop Materials</i>
Percent moisture	Hops
Percent extract	Insect count
Laboratory wort	Percent moisture
Soluble nitrogen	Alpha acids
Free amino nitrogen	Beta acids
Diastatic power	Hop extracts
Alpha amylase	Alpha acids
Protein	Beta acids
	Isomerized hop extracts
<i>Adjuncts</i>	Iso-alpha acids
Adjunct cereals	Rho-iso-alpha acids
Moisture	
Oil	
Enzyme conv. yield	<i>Water</i>
Protein	Turbidity
Ash	Conductivity
Adjunct syrups	Hardness
Clarity	Alkalinity
Color	Dissolved solids
Percent solubles	pH
Percent	Individual minerals
Fermentable	Organic matter
pH	Trihalomethanes
Protein	Chloroform
Ash	Nitrates

Analysis of the Finished Beer

Most of the measurements made on the finished beer are done by the Q.A. lab. The laboratory measurements done can be put in two categories. One group is designed to describe the changes that occur during fermentation. This helps describe, of course, how closely the brewing process follows its plotted course. The other group of measurements deals with beer attributes that are perceivable by the consumer. Table 3.13 gives a typical finished beer analysis.

**Table 3.12 : Analysis Done by Quality Assurance on
"in Process" Wort and Beer**

<i>Chemical Tests</i>	
Wort	Beer
Specific gravity	Specific gravity
Extract	Degree of fermentation
Iodine test	Total acidity
pH	Color
Total acidity	pH
Color	Protein
Protein	Fermentable sugars
Reducing sugars	Free amino nitrogen
Free amino acids	Dissolved oxygen
Viscosity	
<i>Microbiological Tests</i>	
Beer	Yeast
Cultured samples	Microscopic
Bacterial cultures	Cell morphology
Wild yeast cultures	Viability stains
Direct yeast counts	Bacteria search
Cleaning checks	Percent budding
Air sample cultures	Culturing
Stained slides	Differential (bact.)
Fermenter beer	Differential (yeast)
Filtered beer	Measuring
Suspect cultures	Total cell conc.
	Total yeast solids

Table 3.13 : Analysis Done by Quality Assurance on Finished Beer

Air (O ₂) content	Original extract
Alcohol content	Final extract
Calcium/oxalate content	Final degree of fermentation
Clarity, fresh	Foam retention
Carbon dioxide	Iodine reaction
Clarity, punished	Iron
Color	Iso-alpha acids
Copper	pH
Diacetyl	Protein
Final specific gravity	Sodium
Flavor evaluation	Sulfur dioxide

Flavor Measurements

In a modern brewery, the quality Assurance team is the unit that must determine, by analysis and tasting, if a beer is within its total specification range. It must report its findings to top management; it must stand prepared to consult and to lend a hand when corrective action is called for. As noted earlier, it must taste the beer as a critical consumer would.

Tasting beer

Flavor quality of any beer is its most important attribute. Evaluation of the flavor quality of a beer is without doubt the most important analytical procedure done by the Quality Assurance group.

Tastable Beer Defects

Many flavor defects that occur in beer can be recognized by tasting more easily than by any analytical procedure. They can be detected so quickly by tasting that frequently a problem discovered by tasting will be resolved before the chemical analysis is completed. Compounds that are easily identified by taste are:

Diacetyl

It is detectable in beer at about 0.02 ppm. It is able to subtly alter the blended character of a beer at about 0.1 ppm; it contributes a butter-like aroma at this level. At levels approaching 0.2 ppm, it becomes unpleasant; a sharp and cheesy aroma is quite apparent and the unblended character of the beer makes the taster aware of too much bitterness.

Metallic Tastes

Iron and copper have long been recognized as being capable of causing metallic taste in beer. Iron at 0.1 ppm and copper at 0.2 ppm will cause beer to have a metallic flavor. The iron will taste "rusty" and the copper tastes just as a piece of copper in the mouth does. As time passes this metallic taste will disappear and a haze will develop in the beer. These polyvalent metals are able to oxidize susceptible polyphenolic molecules which condense to form larger molecules and ultimately become so large that they will react with peptide molecules, become insoluble and precipitate.

Aluminum at about 2 ppm tends to produce a white haze. This is largely the reaction product of the aluminum ion and large peptides which complex and become an insoluble white precipitate. Unprotected aluminum cans, due to the magnesium present as an alloying metal, cause hydrogen gas to evolve as beer dissolves the can metal. The hydrogen gas reacts with traces of sulfhydryl material to form some mercaptan-like compounds. The result is not pleasant. Low levels of pure aluminum have a pleasing taste.

Some metal ions will not react with polyphenolic material to any significant extent. Lead, nickel, tin, vanadium and molybdenum react slightly. Tin and nickel have very harsh, bitter, metallic tastes.

High Air Beer

When beer contains more than 1.0 ppm of oxygen, its shelf life is impaired and the beer develops old oxidized taste considerably sooner than with only 0.4 ppm of oxygen present. If oxygen is introduced at a level of 2 ppm the beer has essentially no shelf life. It will taste dull shortly after pasteurization. After about 3 days at room temperature, the beer will begin to take on a red (foxy) hue and will taste dull, slightly sweet with no hop character. Yeast can compete successfully with beer polyphenols for oxygen. If too much oxygen gets in the yeasted beer, budding might be triggered.

Light Struck Beer

The thiol that is produced in light struck beer is extremely potent, being detectable at less than 0.1 parts per billion. When it becomes necessary to check the capability of bottles or of bottle cartons for light resistance, a flavor panel can quickly detect this sun struck odor which indicates inadequate protection. Exposure to about 50 foot candles of white fluorescent light gives translatable data on the light resistance of glass packages. This intensity roughly equals that found in a well lighted display case.

Old, Oxidized Beer

Earlier the oxidation brought about by oxygen was discussed. It is not the same oxidation as old, oxidized beer. The presence of high levels of oxygen triggers many oxidation reactions in the beer, some perhaps that normally would not take place for some time under normal low oxygen conditions.

When low oxygen content beer is stored at room temperature (22°C) the following progressive changes take place with time. As the beer begins to age, it develops characteristic old flavors as the polyphenolic molecules oxidize. The hops, too, undergo some oxidation, losing their aspect of freshness. The beer continues to undergo several progressive aging changes in flavor. Sweetness increases then disappears, burnt and caramel-like flavors increase, hop bitterness changes and then disappears, fatty aldehydes develop producing papery to woody odors. Finally after about three years this beer will begin to resemble the high air beer. Only modest changes take place until about 90 days; most brewers want their products to be consumed before then.

Medicinal Odors

Some wild yeasts produce odors that could perhaps be called medicinal. They are sometimes called “phenolic” but when present as the result of a wild yeast infection, the “phenolic” odor is unclean and complicated by the presence of other odorous wild yeast by-products as well. Medicinal odors appear to be related to the chlorphenol type of compounds. *Ortho*-dichlorophenol is detectable in beer at 2 parts per billion; it is unpleasant at about 6 ppb. Today so much chlorination of water is done that the brewer should taste his water daily to make sure that it is free from such odors. If it is not, he should resort to the use of activated charcoal to remove the chlorphenols and any other organo-halides that might be present.

Grainy, Harsh, Astringent, Bitter

As pointed out this kind of bitterness can develop from several causes. Too high a pH in the mash or wort, metal imbalance, the use of malt with high phenol oxidase activity, a high level of yeast autolysis could all contribute to such a condition. Frequently when some factor has unbalanced the blend of flavors in beer, harsh bitter flavors stand out. Other flavors which tended to subdue them are no longer functioning.

Flavor panels have trouble dealing with such flavor changes. The grainy, harsh, bitter flavors are not alien in beer. They are tastable in beer most of the time, but they are held in check. The panelist will have no trouble recognizing their taste disorder; he will be hard put to suggest a cause or cure.

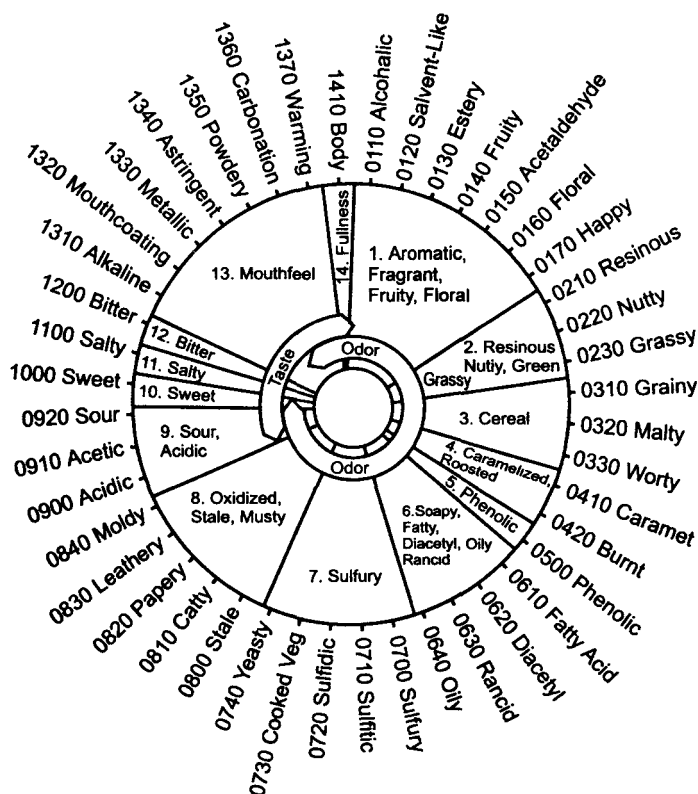


Fig. 3.3 : The flavour wheel

Flavor Depression

A flavor panel encounters this flavor influence much more frequently than in the past. It is most frequently caused by the presence of some organic polymeric (plastic) material that will absorb or dissolve certain of the beer constituents into it. This is quite noticeable when beer is placed in a polyethylene container, for example. The polyethylene will absorb esters from the beer, leaving it dull and depressed in flavor. Many vinyl formulations will do the same as will virtually every polymer. They vary in their specificity for beer components.

A flavor panel must screen the materials that come in contact with beer. Can liners, crown liners, tank liners, etc. should all be

given representative exposure to beer and tasted by a flavor panel looking for harmful flavor effects before they are cleared for use.

Fig. 3.3 is a reproduction of the flavor wheel. This group has done an admirable job of cataloging the various flavors encountered in beer. The figure summarizes all the sensory factors involved in flavor evaluation by nose and mouth. Those purists who feel that clarity and color of a beer should be integral parts of its flavor portrayal will suffer some minor disappointment with this wheel.

Acetone and Butanol

History

The acetone-butanol fermentation has a long history as a successful industrial fermentation process. The earliest work on this fermentation was performed by Pasteur in 1862, who investigated the production of butanol from lactic acid and calcium lactate.

Current Production and Uses

Currently, butanol is manufactured from ethylene and triethylaluminum and is used primarily in the manufacture of lacquers, rayon, detergents, brake fluids and amines for gasoline additives. It can also be used as a solvent for fats, waxes, resins, shellac and varnish. Acetone is a by-product from the manufacture of phenol from cumene, a by-product of oxidation cracking of propane, or can be manufactured by chemical reduction of isopropanol. Acetone is used mostly as a solvent for fats, oils, waxes, resins, rubber plastics, lacquers, varnishes and rubber cements.

Proposed Uses

The acetone-butanol fermentation currently has potential because butanol has many characteristics which make it a better liquid fuel extender than ethanol, now used in the formulation of gasohol. Three of the more important characteristics which make butanol a better liquid fuel extender are : (1) its low vapor pressure, (2) its low miscibility with water and (3) butanol, unlike ethanol, is completely miscible with diesel fuel even at low temperatures (Table 4.1).

Although economically attractive when based on waste-type materials such as cheese whey, the acetone-butanol fermentation

has a number of drawbacks which must be addressed before any attempt for commercial production is made. The major one is the very low levels of acetone and *n*-butanol obtained in the final fermentation broth. With current technology, such levels are only 0.7 and 1.4% (w/v), respectively. This results in the need for large vessels for fermentation and an energy-intensive distillation recovery of the solvents. Sterilization of large volumes of media is also highly energy intensive. Additional difficulties with this fermentation include the need for strict anaerobic conditions and delicate culture maintenance and propagation.

Fermentative Production

The organism *Clostridium acetobutylicum* will ferment a variety of carbohydrates such as lactose, glucose, xylose, fructose, arabinose, galactose, maltose, mannose, starch and sucrose and produce a variety of organic solvents including butyric and acetic acids, butanol, acetone, ethanol, carbon dioxide, hydrogen, isopropanol, formic acid, acetone (acetylmethylcarbinol or 3-hydroxy-2-butanone) and a yellow oil, which is a complex mixture of higher alcohols, higher acids and esters. Butanol, acetone and ethanol are normally considered to be the principal products of this fermentation.

Raw Materials

Starch

The production of acetone and butanol by the Weizmann process utilized starch as substrate. *C. acetobutylicum* possessed amylolytic and saccharolytic enzyme activities required to hydrolyze gelatinized starch to glucose and maltose. Concentrations of corn of 8 to 10% (5 to 6.5% starch) were readily utilized; average solvent yields of 38.0%, based on sugar fermented were reported. Wheat, milo and rye served equally well. Potatoes could not be handled at high concentrations because of viscosity problems. Normally, the germ and bran were removed from the grains prior to milling in order to recover these valuable by-products. The grain mash was first gelatinized for 20 min at 65°C and then sterilized for 60 min at 102 °C. This cooked mash was cooled by means of double-pipe heat exchangers to 35 °C as it was pumped through steam-sterilized lines to fermenters of 250 000-2000 000 l capacity. The fermenter was inoculated either during or

Table 4.1 : Characteristics of Chemically Pure Fuels

<i>Fuel</i>	<i>Molecular weight</i>	<i>Specific gravity</i>	<i>Boiling point (°C)</i>	<i>Vapor Pressure at 37.7°C (p.s.i.)</i>	<i>Combustion energy (kJ kg⁻¹)</i>	<i>Latent heat (KJ Kg⁻¹)</i>	<i>Solubility (parts in 100 parts H₂O)</i>	<i>Stoichiometric air-fuel ratio</i>
Methanol	32	0.79	65	4.6	23 864.8	1170.0	□	6.5
Ethanol	46	0.79	78	2.2	30 610.6	921.1	□	9
Butanol	74	0.81	117	0.3	36 681.0	432.6	9	11.2
Octane	114	0.70	210	1.72	48 264.5	360.5	insoluble	15.2
Hexadecane	240	0.79	287	3.46	47 264.3	—	insoluble	15

after the filling process with 3% (v/v) inoculum from a 24 hour culture tank. In some cases, 30 to 40% of the total aqueous volume of the mash was provided by stillage from which neutral fermentation solvents from previous fermentations had been stripped. This so-called slopping-back procedure added certain nutrients to the medium which increased yield, aided in foam control and reduced the amount of stillage which required evaporation and drying for feed supplement preparations.

The fermentations which lasted 2 to 2.5 days, passed through three phases. The first was characterized by rapid growth, production of acetic and butyric acids, and evolution of carbon dioxide and hydrogen, primarily the latter. The pH, initially 6.0 to 6.5, decreased during this phase to a constant value determined by the particular buffering capacity of the substrate and medium. The 'titratable acidity' however increased to a maximum which defined the end of the first phase. The second phase, called the acid break, began with a sharp decrease in the titratable acidity' after 12 to 14 hours. The organisms had begun to convert the acidic products to neutral solvents. Gas production increased, a greater proportion being carbon dioxide than in the earlier phase of the fermentation. During the third phase, the rates of gas and solvent production decreased until the cells autolyzed. The corn substrate fermentation ended at between pH 4.2 and 4.4.

Molasses

Scientists described the use of a variety of other *Clostridium* species used for fermentation of sugar in molasses. The fermentation process was similar to that described above for starch except that the mash could be sterilized at a lower temperature, the fermentation was conducted at 31 to 32 °C, cleaning of tanks and lines was easier, higher concentrations of sugars could be used and a greater ratio of butanol to acetone plus ethanol was produced. It was necessary to add nitrogen and phosphate nutrients to molasses fermentations (e.g. 1.0 to 1.4% ammonia based on sugar present). The ammonia was usually added to maintain the pH at 5.6 after the 'titratable acidity' dropped the pH from an initial value of 6.5 during the first 16 hours of fermentation. The molasses production medium contained 6% sugar calculated as sucrose; average solvent yields of 30.0% were reported.

Cheese Whey

Since *C. acetobutylicum* has the capability of metabolizing lactose, it was possible to utilize cheese whey as a feedstock for the acetone-butanol fermentations. Cheese whey, the fluid which remains following separation of the curd when converting milk into cheese, contains about 6% lactose by weight and in the order of 1% protein, depending on the type of cheese manufacture. The level of sugar in cheese whey coincided with that required in the medium for the acetone-butanol fermentation. Cheese whey preparations from which approximately 75% of the protein was removed by simple heat denaturation, acidification or ultrafiltration have proven to be suitable fermentation substrates.

The fermentation of lactose in cheese whey produced some interesting modifications in the product distribution and yield. The classical solvent ratio produced by *C. acetobutylicum* from corn mash was 2 to 1 butanol to acetone. With pure glucose as substrate, a ratio of 2.7 to 1 was obtained. Under the same conditions, substitution of lactose for glucose resulted in a product stream in which the relative proportion of butanol to acetone was 2.9 to 1. The fermentation of cheese whey resulted in a strikingly different set of product concentration profiles. For instance, the normal intermediary build-up of acetic and butyric acid was not observed and the final ratio of butanol to acetone was 9.7 to 1. Others also reported a 10 to 1 butanol to acetone ratio in fermentation of an ultrafiltrate of cheese whey. Fermentations on cheese whey from which 80% of the protein was removed by heat precipitation and ultrafiltration yielded butanol to acetone ratios of 12.3 to 1 at 37°C. Others also obtained 10 to 1 ratios of butanol to acetone with ultrafiltered cheese whey, using one particular strain of *C. acetobutylicum*, but that result was not consistent under all experimental conditions.

Jerusalem artichoke

An assortment of raw materials such as beet molasses, wheat, rice, horse chestnuts, potatoes and Jerusalem artichokes, have also been used in this fermentation. The French government was recently making a concerted effort to produce quantities of butanol for emergency fuel purposes from Jerusalem artichokes. The potential for utilizing not only fructose from tuber-derived inulin, but also the

structural carbohydrates in the vegetative stalk of the plant has been investigated. Until anthesis, the inulin content of the stalk and leaves is greater than that of the tubers. The protein content of the leaves is great enough to offer substantial by-product credit from extracted leaf protein. Butanol to acetone ratios of 2.75 to 1 (w/w) from acid hydrolyzed inulin from Jerusalem artichoke tubers have been obtained.

Lignocellulosic hydrolysates

C. acetobutylicum can anaerobically ferment different carbohydrates into a spectrum of products including *n*-butanol, acetone, ethanol, butyric acid, acetic acid and acetoin. Hydrolysates of lignocellulosics, *i.e.* wood, paper, crop residues, *etc.*, primarily contain glucose and cellobiose as well as galactose, mannose and the pentose sugars such as D-xylose and L-arabinose, from the hemicellulose fraction. Glucose, cellobiose, mannose and arabinose have been shown to be fermentable. Galactose and xylose were utilized poorly and primarily yielded acids. Others also noted that under the conditions of their study, better solvent production was obtained from sugars arising from the *erythro* configuration (glucose, mannose, arabinose) as compared to the *threo* configuration (xylose, galactose).

Workers reported a significant increase in acetone and butanol production occurred when *C. acetobutylicum* was grown on D-xylose in the presence of 1 g l⁻¹ acetic acid or 3 g l⁻¹ butyric acid added prior to inoculations. Others reported very low conversion rates of the pentose fraction from steam exploded aspen wood by *C. acetobutylicum*, but this may have been due to the extreme sensitivity of the organism to furfurals. Scientists reported an average of 30.6% yield, based on sugar fermented, of solvents with normal product distribution when fermenting corn cob saccharification liquors, composed primarily of xylose, with *C. butylicum* (NRRL 594). Others also reported similar product ratios and yield using *C. butylicum* for the production of acetone and butanol from the wood sugars in waste sulfite liquor which is primarily xylose, following lignin precipitation with calcium ions. Xylose utilization by *C. acetobutylicum* was also shown to be improved with concentrations of up to 10 g l⁻¹ calcium carbonate.

Current and Potential Processes

South African operation

The commercialized production of acetone and butanol is still carried out by the fermentation of cane molasses with the organism *C. acetobutylicum* in South Africa by National Chemical Products Ltd., Germiston, South Africa. The fermenters used there are 90 000 l working volume and considerable detail has been given about the operation of the plant. Distillation is used to recover solvent from the acetone-butanol fermentation broth, which contains approximately 2% (w/v) solvents. The fermentation broth is fed at a constant rate to the top of a beer still containing 30 perforated plates, from which an approximately 40/60 (wt %) solvent/water stream was removed overhead from slops (water and stillage).

The mixed solvents are then separated by batch fractionation. Acetone, butanol and a mixture of ethanol and isopropyl alcohol (formed by reduction of acetone in the beer well) are obtained as separate fractions. The butanol is dried by removal of the distillate through a decanter. A high boiling fraction containing higher alcohols, esters and organic acids is also obtained. The slops contained bacterial cells rich in riboflavin and B vitamins which are concentrated, dried and shipped around the world as animal feed. Rumumco Ltd. of Burton-on-Trent in Great Britain has handled the distribution of the dried products from the South African NCP operation.

Cheese whey

An anaerobic waste treatment stage which would generate methane was considered in the whey analysis and was retained in this analysis for partial supply of energy requirements by utilization of minor fermentation product streams such as acetic acid, butyric acid, acetoin and other organics. Since separation and compression costs for hydrogen were not considered in the original equipment and operating analysis, allowance for the anaerobic conversion to methane of 25% of the carbon dioxide and 100% of the hydrogen produced in the *C. acetobutylicum* fermentations by methanogenic bacteria in the waste treatment stages would replace approximately 10% of the product recovery costs. It was assumed that the liquid

wehly was available for the cost of trucking an average 160 km from cheese plants to a central fermentation facility at current trucking rates.

This economic study provided a preliminary estimate ($\pm 25\%$) and was based on the flow sheet from acetone-butanol fermentation plant design in Figure 4.1. The following characteristics were used : (a) about 45×10^6 kg per year solvent ; (b) location in the state of Wisconsin; (c) 300 day per year operation, with 6 hour staging of fermentations; (d) costs reported are end 1982 values; and (e) protein is recovered through heat denaturation and rotary-drum filtration.

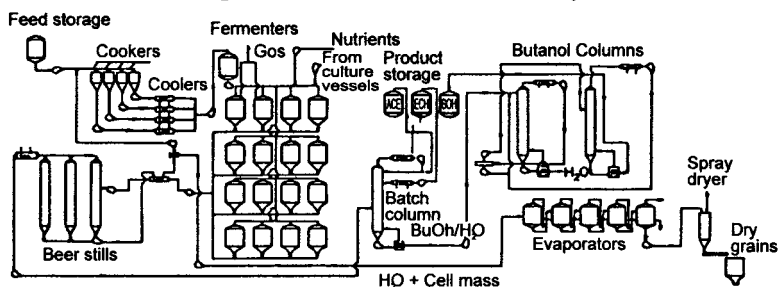


Fig. 4.1 : The process flow-sheet for the acetone-butanol fermentation

Lignocellulose hydrolysates

A process for production of butanol from cellulose waste has been given and includes a discussion of the impact of genetic manipulation of *C. acetobutylicum*. The yields predicted were thus greater than currently feasible and the fermentation temperature was twice the current optimum.

Once subjected to various types of pretreatment, the hemicellulose and cellulose fractions of wood residues would undergo enzymatic hydrolysis and would yield the monomeric form of the corresponding sugars, primarily xylose and glucose. Both pentose and hexose sugar solutions could be fed to a fermentation vessel where *C. acetobutylicum* would ferment those sugars to acetone, butanol and ethanol. Hydrogen and carbon dioxide would also be products of the fermentation.

Based on sawdust availability of 250 ton day^{-1} and an average composition of 25% lignin and 65% carbohydrate (dry basis), a plant operating 300 days per year would produce 15 000 ton per

year of lignin and 13.6×10^6 l per year mixed solvents (acetone + butanol + ethanol). This estimate for solvents production was based on available solvent yield data for oak hydrolysates. Utilizing as an estimate the hydrogen and carbon dioxide data for blackstrap molasses and the current selling prices for the relevant commodities, an income estimate per ton of residue was prepared.

Impact of Added Fermentative Production

Basis : Available Wood Residues

According to a recent report, the supply of wood residues available for conversion into fuels and chemicals was estimated at 179 million dry tons in 2003 with the following relative distribution: 163 million tons from commercial logging residues and 16 million tons from primary wood manufacturing residues, including bark. Although the composition of these residues is species specific, the following average figures (on a dry basis) will be assumed in our projections for reasons of simplicity lignin, 25%; carbohydrate (cellulose + hemicellulose), 65%. Based on the previous figures and assuming reasonable product yields in the proposed conversion scheme, the data were then calculated.

Petrochemical Displacement

The possibility of achieving total replacement of the petrochemicals used for phenol, butanol, acetone and ethanol manufacture and still stimulate the creation of new domestic markets, or the development of new export markets, certainly points to a promising future for wood as a source of chemicals. Alternatively, if the butanol produced from wood biomass is envisioned as being utilized as a gasoline extender (gasohol production), the 3.8 billion pounds could replace 5% of the current annual gasoline consumption. If used in the same way, the ethanol produced could add almost another 1% to this consideration.

While the production potentials discussed above may represent valid maxima today, economically collectable quantities of wood residues would probably be limited to those from primary manufacturing, within limitations of current technology and economy. Thus, the practical potentials would be an order of magnitude lower and in rather good balance with current production (demand). It is appropriate, then, to examine the effect

of reasonable displacements upon demands for petrochemical feedstocks.

Phenol

Suitable lignin products could reasonably satisfy 25% of the phenol requirements of the one billion pounds of phenolic resins produced annually. Such displacement would represent about 175 million pounds of phenol. Production of such an amount of phenol requires about 210 million pounds of benzene and 113 million pounds of propylene. These latter two chemicals are primarily derived from petroleum. Note also that production of phenol and its precursors consumes considerable amounts of energy, which are unfortunately not very readily quantifiable.

Butanol

If some 18% of the butanol derivable from primary wood residues were to be used to displace the butanol currently produced by the petrochemical industry, this would relieve propylene demand by about 420 million pounds per year. Thus, this propylene, now consumed in butanol production, would be available for other feedstocks or for use in gasoline, as an octane improver. (While propylene is one of the principal petrochemical building blocks, 55% of production goes into gasoline). Energy expended in the butanol manufacturing scheme would be saved in addition.

Acetone

Similarly, the 1.8 billion pounds of acetone from primary residues could displace 50% of the petrochemical acetone, manufactured by isopropanol dehydrogenation (the other 50% is a by-product of phenol production from cumene hydroperoxide and is not truly displaceable), and the ethanol could approximately halve the current demands of synthetic ethanol upon supplies of ethylene, our most important petrochemical building block. Again, the energy now consumed in manufacture of the displaceable acetone and ethanol (and their precursors) would go on the credit side of the ledger for the hypothetical process.

The preceding analysis, although simplistic in nature, indicates that the butanol, acetone, ethanol and phenol quantities

potentially derivable annually from primary manufacturing wood residues are equivalent to a total of about 6 billion pounds of petrochemicals. Although information on the energy required to produce these chemicals from petroleum is not readily available, it can be safely stated that at least a like quantity of crude petroleum will be replaced and can be rerouted to other uses. The energy requirements for the proposed alternative process (wood \rightarrow chemicals) are also rather speculative at this time; very preliminary calculations show a total energy consumption of about 150 000 BTU to derive one gallon of mixed solvents and 10 pounds of lignin from the wood residue. It seems reasonable to assume at this stage that the energy requirements for both processes will be of the same order of magnitude and consequently the alternate process spares at least as much petroleum as the 6 billion pounds of chemicals produced. Since wood residues can be burned simultaneously to supply energy to the proposed bioconversion scheme (as opposed to what happens, for instance, in a petroleum refinery), the potential petroleum displacement of this project can ultimately be significantly enhanced relative to the 6 billion pounds figure cited earlier.

Hydrogen and Carbon dioxide

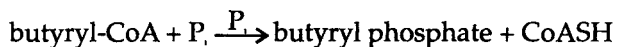
The total weight of the gases formed during a fermentation exceeds that of the solvents. In fact, only 38% yields of neutral solvents based on substrate consumption can be expected from the fermentation; the remainder of the fermentable sugars end up as carbon dioxide and hydrogen. Since the most important factor in the economics of the process is substrate cost, utilization of these by-products should be considered in an integrated process. The gases can be separated by selective absorption of carbon dioxide in a liquid amine or by using a membrane separation. Pure hydrogen can be used as a fuel, in fuel cell application to generate electricity or as a reductant for chemical reactions. Examples of the latter are methane formation by methanogenic bacteria, synthesis of ammonia by catalytic reaction with nitrogen at high pressure and temperature, and methanol production by reaction of either CO_2 or CO with hydrogen. Prior to the close of the acetone-butanol fermentation industry, Commercial Solvents produced ammonia and methanol from fermenter off-gases. Using current technology, the latter has been shown to yield an incremental rate of return of

25 to 30%. Carbon dioxide could be used for dry ice production or for oil recovery operations where a market existed.

Phosphate Limitation

Workers have described batch fermentations of *C. acetobutylicum* which demonstrate the regulation of butanol and butyric acid production. The biochemical reason for the observed regulation appears to lie in the involvement of butyryl phosphate in the energy metabolism of the cell. During the acid phase (while sufficient phosphate is present), butyrate is primarily formed by reaction of two enzymes which occur in *C. acetobutylicum*.

(i) phosphotransbutyrylase :



(ii) butyrate kinase :



Phosphate limitation does not allow the formation of butyryl phosphate. With the resulting accumulation of butyryl-CoA, butyraldehyde dehydrogenase is induced or activated by an unknown mechanism and catalyzes the formation of butyraldehyde which is further reduced to butanol.

Addition of acetic and butyric acids in continuous cultures and in batch cultures improved butanol productivities. Concentrations of butyric acid in the nondissociated form in the fermenter have been correlated with the acid shift. Components which accompany the fermentable carbohydrate from various practical sources influence the distribution of fermentation products. For instance, calcium is a low molecular weight component of cheese whey, which is capable of complexing phosphate and which has been historically used to improve butanol yields in the fermentation of grains, molasses and wood wastes.

Agitation Control

The two phases of the *C. acetobutylicum* fermentation described above are manifestations of the different ways in which the organism regenerates its biochemical reducing potential. During the acid phase, this is done by production of hydrogen gas. The reaction is catalyzed by a ferredoxin-linked hydrogenase, which also has the ability to shunt the reducing power to the cofactor,

NAD. Excess reducing power in this form can be disposed of by reducing butyryl-CoA to butanol in two steps in the second phase of the fermentation. This equilibrium between H_2 and the reduced $NADH_2$ has an important influence on solvent production. Since the hydrogenase catalyzes a reversible reaction, an increase in the concentration of dissolved hydrogen would prohibit the production of more hydrogen and encourage the formation of neutral solvents.

Agitation and gas head space pressure are two means of controlling dissolved gas concentrations. Others has shown that greater agitation rates favored the simultaneous production of hydrogen and butyric acid in *C. acetobutylicum* fermentations. Inversely, hydrogen supersaturation in the medium, favored at lower agitation rates, e.g. 25 r.p.m., enhanced butanol formation. Butanol and ethanol productivity were also increased under pressurised conditions (15.2 p.s.i.g.). Since the butyric acid productivity peaked earlier in highly agitated fermentations than at lower agitation rates, overall fermenter productivity would be improved by a combination of moderate to high agitation (e.g. 300 r.p.m.) during the acid phase followed by low agitation during the solvent phase.

Extractive Fermentation

Butanol recovery by extraction has represented an area of considerable research. Not only will extractive fermentation increase fermenter productivity, the toxicity of butanol to *C. acetobutylicum* may be controlled. One type of extractive fermentation which has been studied is one in which the extraction involves contacting a recycle stream of fermentation broth outside the fermenter. Alternatively, approaches which have shown partial success are *in situ* extraction systems. Corn oil, paraffin oil, kerosene and dibutylphthalate have been examined and found not to affect cell growth. Corn oil did not affect the productivities and conversion yields of acetone and butanol in an *in situ* fermentation; the total concentrations of acetone and butanol in both phases were 10.6 and 19.6 gl^{-1} respectively. Activated carbon has also been used to increase the overall solvent concentrations during *in situ* fermentations.

Using *n*-butyl *n*-butyrate, which could be formed directly from two of the fermentation products, showed the ester extractant exhibited the following distribution coefficients with five

fermentation products : *n*-butanol, 3.50; butyric acid, 2.15; ethanol, 1.20; acetone, 0.76; and acetic acid, 0.04. In a controlled fermentation in which a 4 to 1 volumetric ratio of aqueous phase of ester was used, 19 g l⁻¹ butanol and normal distribution of other fermentation products were obtained.

Experiments reported with polypropylene glycol extractant produced changes in water activity which inhibited cellular functionality.

Continuous Fermentation

Fermentations operated continuously or semi-continuously could improve reactor productivity. Volumetric butanol productivity could be increased three-fold in continuous culture over that obtained in batch culture (2.5 g l⁻¹ h⁻¹ versus 0.8 g l⁻¹ h⁻¹). It was also shown that specific productivities of acetone and butanol increased with dilution rate to maximum values of 0.3 and 0.2 g g⁻¹ cells h⁻¹, respectively, at the dilution rate of 0.22 h⁻¹; above that dilution rate, the fermentation favored butyric acid production.

The use of continuous culture has also been employed for studies of nutritional and environmental factors which affect solvent production. Some studied the fermentation under nitrogen 'limitation; others studied the fermentation under phosphate limitation. The latter two groups have also used continuous culture to study the effect of pH and of butyrate concentration on solvent production. Near theoretical conversion of glucose into solvents has been obtained using continuous culture, but as in batch culture, butanol toxicity limits high product concentrations and volumetric productivities.

Immobilization

The continuous production of butanol has been reported from immobilized *C. acetobutylicum* and *C. butylicum*. Spores and vegetative cells were immobilized in calcium alginate gel and studied under what were termed non-growth conditions. The productivity of these cells was found to be reasonable (1.0 g butanol l⁻¹ h⁻¹), but because butanol toxicity rapidly reduced cell activity within the immobilization matrix, extensive investigation will be required to establish economical working conditions for such a process.

Biochemical and Genetic Developments

Workers describe variations in the morphology of *C. acetobutylicum* during different stages of the fermentation process depending upon the strain of organism used and the formulation of the medium. An encapsulated form predominates during neutral solvent production. Others have described some of the ultrastructure of *C. acetobutylicum*. Fundamental information derived from such studies has been used to improve butanol tolerance in *C. acetobutylicum*.

Toxicity of Products

For each fermentation product, there exists a threshold concentration below which no growth inhibition occurs, and above which a linear decrease in growth rate is observed. These values are given in Table 4.2. The fermentation end products are in dilute solution primarily because of butanol toxicity. Research has been aimed at gaining a fundamental understanding of alcohol toxicity in fermentations and at investigating potential means of overcoming the problem. Aliphatic alcohols were found to inhibit cell growth cytoplasmic membrane functionality in *C. acetobutylicum*. Concentrations on the order of 1 M ethanol, 0.1 M butanol and 0.01M hexanol were found to inhibit by 50% the cell growth rate, the active nutrient transport process and the membrane-bound ATPase activity. The intensity of the alcohol-induced effects was proportional to the hydrophobicity of the aliphatic alcohols at any given concentration.

Table 4.2 : Concentrations of Fermentation Products Added to Active Cultures which were Inhibitory to *C. acetobutylicum* (ATCC 824)

Product	Concentration below which growth was not inhibited		Concentration at which growth was inhibited 50%	
	(M)	(g l ⁻¹)	(M)	(g l ⁻¹)
Butyric acid	0.02	1.7	0.07	6.0
Butanol	0.05	3.7	0.15	11.0
Acetic acid	0.04	2.5	0.13	8.0
Ethanol	0.25	11.6	1.10	51.0
Acetone	0.36	20.9	1.45	84.0

Alteration of phospholipid composition of the cytoplasmic membrane of *C. acetobutylicum* by selective incorporation of specified

fatty acids was accomplished by blocking fatty acid synthesis in biotin deficient media. The cell membrane was enriched in oleic acid or elaidic acid when supplemented into respective media at 10 mg l^{-1} . Cell growth and membrane functionality (ATPase activity) were inhibited by *n*-butanol to a lower degree in these cells than in cells grown on normal soluble medium. Addition of 10 mg l^{-1} of oleic acid (18:1 *cis*) or elaidic (18:1 *trans*) acid to a biotin deficient culture medium has also shown the potential for obtaining greater butanol levels in the fermentation.

The economic impact of increasing the butanol concentration in the fermentation broth to 20 g l^{-1} would be significant. The calculations were based on recovery of the azeotrope as the overhead product from feed of saturated liquid at the given fermenter concentrations. The calculations showed that to increase fermenter concentration from the typical 1.2 to 1.9% (w/w) would halve the energy consumption for distillation. Concentrations greater than the latter would have diminishing impact on distillation costs.

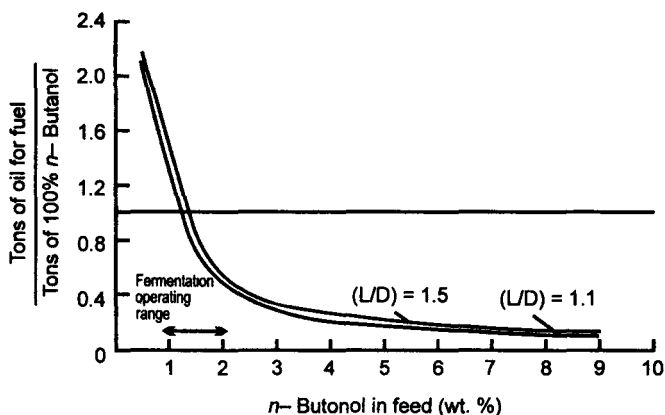


Fig. 4.2 : Energy requirements for *n*-butanol recovery by distillation (Phillips and Humphrey, 1983)

Genetic Developments

The production of solvents during the acetone-butanol fermentation is not only affected by product inhibition, but may also be inhibited by the production of an autolysin. During the fermentation of a molasses medium, it was found that an autolysin

was released toward the end of the exponential growth phase and accompanied the lysis of the culture. The exact role of the autolysin in the fermentation process is open to speculation; it may be a factor accounting for the low level of solvents achieved.

Anaerobic fermentations which operate at high temperatures are of particular interest for the production of fuels and chemicals, because process heat can help in recovering volatile products, contamination risks are reduced and oxygen transfer limitations are not critical. The utility of *C. acetobutylicum* would be greatly improved if thermophilic characteristics were incorporated by genetic or in *vitro* recombinant DNA technologies. Several thermophilic clostridia are particularly promising for the production of chemicals from biomass: (1) *C. thermoaceticum* for the production of acetic acid; (2) *C. thermosacchrolyticum* for butyric acid production; (3) *C. thermocelum* for its ability to degrade cellulose; and (4) *C. thermohydrosulfuricum* for its high yields of ethanol. Genetic manipulation of *C. acetobutylicum* and of the thermophilic clostridia would be possible were a suitable vector for transfer of recombinant DNA available. The capability for transformation and regeneration, of *C. acetobutylicum* protoplasts and the ability to enumerate *C. acetobutylicum* using conventional microbiological techniques for aerobes have been demonstrated. The latter has been made possible by incorporation of membrane particles from facultative anaerobes into the *C. acetobutylicum* growth medium. These developments are certainly encouraging in this regard.

5

Cheese

There is no recorded evidence for tracing the origins of cheesemaking. It is believed that rudiments of cheesemaking originated in the Middle East. People who inhabited the Tigris-Euphrates Valley in the dawn of history, and who later migrated and settled in Western Europe and South and Central Asia were mainly pastoral nomads who moved in search of pasture and water for their cattle. Milking being an integral part of herding cattle, milk and milk products probably became an essential part of the nomad's diet. These early people carried water in sheep stomachs and animal skin bags, and it is possible that other fluids like milk were also carried in similar containers. It is not far-fetched to conceive that serendipity and fortuitous combination of natural flora of milk, animal stomachs, the rennin from the glands lining the inner wall of sheep stomach, and warm temperature gave rise to the primordial cheese. Cheese making most likely was spread by these people to South and Central Asia and the land mass north of the Mediterranean as they sojourned through these areas.

Early Eastern writings dating as far back as 2000 B.C. support the existence of cheese in the ancient diet and as an item of barter.

The crude beginnings of cheesemaking were elevated to an early semblance of technology by the Romans.

Definition of Cheese

Cheese is the consolidated curd of milk which may be consumed as such with little or no modifications or in other instances may before consumption be moderately or even extremely modified through the introduction of different microorganisms or additives and/or by aging (or curing or ripening) the consolidated curd mass under controlled conditions for varying periods of time.

Reasons for Converting Milk into Cheese

The basic reason for converting milk into cheese is to preserve the valuable nutrients in milk. In other words, cheese is made because this affords a means for converting an easily perishable food, milk, into a stabler product, cheese. The need for such conversion becomes important when a surplus of the perishable primary product, namely fluid milk, is available in abundance during flush seasons of the year.

Another reason for converting milk into cheese is to provide variety in foods. During cheesemaking, fermentation of milk by specific microflora, the accompanying manufacturing steps, and the conditions and length of curing induce changes in body, texture, flavor, color, and nutritive properties to create a wide variety of delicious varieties. The resultant cheeses may be consumed as such or may be used to enhance and fortify the flavor and nutritive properties of other basic food commodities like flour, vegetables, eggs and meat during cooking, or baking, or in salads.

Principles of Preservation in Cheesemaking

Perishability of any food product is dependent upon the kind and rate of degradation of the food by microorganisms or enzymes inherent in the food; for example milk lipase induces rancidity if milk is improperly handled. Hence, principles of food preservation involve the prevention or retardation of microbial and enzymatic degradation of foods. Basic principles of food preservation consist of the following:

1. Reduction in the water activity i.e. partial or complete removal of free water or the increase in the concentration of solids.
2. Reduction in available oxygen concentration.
3. Addition of common, edible, "natural" preservatives or condiments such as salt (dry or brine), sugar, syrup, honey and spices.
4. Addition of heat.
5. Decrease in the pH or an increase in titrable acidity.
6. Production of inhibitory metabolites as a consequence of various fermentation reactions by microorganisms.

7. Depletion or removal of food components which can be rapidly metabolized by microorganisms.
8. Smoking.
9. Addition of chemical preservatives.
10. Packaging.
11. Combinations of any or all of the preceding.

1. Removal of Water

Manufacture of cheese represents processes where only a portion of the total moisture in the raw material is removed in the form of whey. Further loss of moisture occurs during curing. This serves twin purposes, namely, the stabilization of the product from *rapid* deterioration and reduction of bulk for ease in storage and transportation. Relative moisture contents of cheeses form the basis of classification of cheese into soft, semi-soft, hard, and very hard varieties. Soft cheeses are generally consumed fresh (Cottage and Baker's cheese) or after relatively short ripening processes (Camembert, Brie, and Bel paese cheese). Cream cheese may be consumed fresh or stored for several weeks although it is unripened and does not undergo appreciable change during this time. The Indian soft cheese, Surti, is preserved by allowing it to float in acid whey. Dry Cottage cheese curd is sometimes given an extended life by storing it in brine.

The manufacturing steps for soft cheese depend on the variety. However, the curd is frequently dipped or otherwise removed from the vat while it is quite soft. The whey is allowed to drain from the curd in hoops or molds of various sizes, or it may be accomplished by harvesting the curd in muslin bags, hanging the bags and allowing gravity to effect whey drainage. In either case, the upper layers of the curd mass compress the lower layers to facilitate greater whey expulsion as well as a more compact body and structure. The ripened cheeses are usually turned or inverted in the hoops and are frequently stacked two or three high in the hoops to insure proper whey drainage and body development.

Semi-soft cheese, i.e. Roquefort, Limburger, and Munster, are also generally not mechanically pressed to expel additional whey from the curd. They are usually dipped when the curd is more firm and also tend to lose more moisture during curing. Hard cheeses

(Cheddar, Emmentaler, etc.) are mechanically pressed to promote whey drainage. Very hard or grating cheese (sometimes called "grana") such as Parmesan and Romano are also mechanically pressed, but the curd has been subjected to additional processing techniques which encourage greater moisture loss. These techniques frequently include a smaller curd particle size and higher cooking temperatures. Additional loss of moisture may occur during brining or dry salting and during curing or aging.

In cheeses that are dry salted before the curd is hooped salt concentration in free moisture within cheese blocks is quite high. This brine exercises a severe osmotic inhibitory effect on microorganisms. In certain cheese varieties, solids are concentrated by heat evaporation of moisture.

2. Reduction in Available Oxygen Concentration

The ability of microorganisms to metabolize and multiply is, in part, determined by the oxidation/reduction (O/R or redox) potential of the substrate. Removal or exclusion of air, in part or in whole, exerts a controlling effect on the growth of many spoilage bacteria and may serve to select for them or against them. Anaerobic bacteria are unable to express growth on or near highly oxygenated surfaces while the growth of aerobic organisms such as *Pseudomonas* and fungi is promoted by such conditions.

The concentration of available oxygen in cheese is governed by three primary factors. First, is the size and shape of the cheese. Secondly, the growth of certain microorganisms may result in a decrease in available oxygen, changing an aerobic microenvironment into one which is anaerobic and subsequently more suitable for the growth of anaerobic organisms. Third, is the mechanical exclusion of air. This concept is an ancient one and is the underlying principle involved in the waxing of cheese or its coating with mineral oil, or the storage of cheese submerged in a brine. These techniques, along with others, have been effectively used to suppress the growth of molds and aerobic spoilage bacteria.

3. Addition of Edible "natural" Preservatives

By far, the most common edible natural preservative is sodium chloride. It has three primary effects in the cheese. First, it contributes its own flavor or saltiness. Second it complements and

accentuates a myriad of subtle flavors in the cheese. Third, in some cheeses, a more important function is that of a preservative. The chemical nature of salt is highly inhibitory to many microorganisms. This inhibition is, at least in part, due to the reduction in A_w . Since salt is ionizable in solution, it increases the osmotic pressure of the system.

In addition to the aforementioned functions, salt exerts a regulatory effect on the ecological flora of both the surface and internal microenvironment of the cheese. In some instances, the concentration of salt will favor the dominance of certain desired types of microorganisms while in other instances salt may tend to exclude the growth of undesirable microflora. During the manufacture of certain surface ripened cheeses, such as the traditional Brick cheese, the exterior of freshly made cheeses is sprinkled or rubbed with dry salt for 2 to 5 days. Because of this, the salt concentration at the surface is very high.

Blue cheese curd is generally subjected to a light application of salt prior to hooping. The surfaces of the subsequent loaves or wheels are heavily salted in brine or dry salted resulting in an initially high salt concentration at the surface, which gradually equilibrates throughout the cheese during ripening. This progressive change in salt concentration dictates the special sequence of fermentation events which characterize Blue cheese.

4. Application of Heat

Heating is the simplest and the most effective way of controlling microbial and enzymatic deterioration of milk. In the manufacture of many cheese varieties, sub-pasteurization heat treatment of milk (about 64°C for 17 seconds) is used to destroy pathogenic bacteria and spoilage microflora and partially inactivate natural milk enzymes. In other instances, fully pasteurized milk is used.

5. Decrease in pH

Most bacteria prefer a growth medium with a pH between 6.3 and 6.8 although some are capable of pronounced growth in much more acidic environments. The fungi are generally more tolerant of acidic conditions, the molds being more aciduric as a group than the yeasts. Most cheese varieties, as well as other cultured dairy products, have a pH of 5.3 or less, plus a comparatively depressed A_w , a large population of live culture organisms, and a significant

"effective" concentration of salt. These attributes tend to exert a controlling effect on the growth of pathogens and spoilage bacteria. Fermentation of lactose in cheese milk by starter cultures yields primarily lactic acid. Accumulation of lactic acid facilitates curd formation (coagulation), moisture expulsion from the curd, suppression of spoilage and pathogenic flora, and toxin production, and promotes the establishment of specific pH and eH ranges within the cheese. These are necessary for regulating microbial and enzymatic reactions which are involved in normal curing and flavor development. If there is proper acid development in the cheese vat, coliforms are suppressed; also the low pH prevents growth and elaboration of enterotoxin by *Staphylococcus aureus*.

6. Fermentation - Use of Starter Bacteria

Starter bacteria are used as acidulating agents in most cheeses. The depression of pH and eH by fermentation products from the metabolic activity of starters inhibits spoilage as well as pathogenic microflora and arrests undesirable enzymatic reactions. Certain strains of *Streptococcus lactis*, a mesophilic starter organism used in the production of many cheese varieties, produce an antibiotic nisin. Nisin inhibits Gram positive, spore-forming bacteria. In Europe, nisin is used to control wild clostridia in cheese milk which cause gas blowing, undesirable butyric acid fermentations and "stink spots" (black or grey patches accompanied by foul odor due to hydrogen sulfide).

7. Removal of Components that are Readily Attacked by Microorganisms

Microorganisms require a carbon source, a nitrogen source, and a supply of minerals and accessory growth factors for normal growth. Of these, the carbon source is the primary target, because these substrates provide the energy required for driving synthetic mechanisms needed in growth also, intermediates from carbon substrates provide the carbon skeleton for the synthesis of cellular components. Depletion of carbon and energy source(s) from a food system, therefore, renders it more stable against rapid microbial deterioration. In cheese making, starter bacteria ferment lactose into lactic acid, and when the whey is drained, almost all of the residual lactose (which is soluble in water) is removed from the curd.

8. *Smoking*

Smoking of cheese performs two functions. Firstly, it imparts the characteristic "smoked" flavor. Secondly, smoking helps to preserve the high quality of the cheese. Smoking causes varying degrees of surface drying depending upon the time and the relative humidity and temperature of the smoking chamber. Smoke also deposits an antimicrobial film of phenolic, crystallic and aldehyde compounds on the cheese surface. Provolone and its sister cheeses (Giganti, Salamini, Bocchini, Mandarini, Provolette, etc.) are among the most commonly smoked varieties. Mozzarella is another Italian cheese which is sometimes smoked. For specific markets, other cheese such as Cheddar and Swiss may be smoked. The natural smoking processing involves the suspending of cheese by cords in the smoke filled interior of a smoke house or the placing of cheese on racks in a smoke chamber. Hardwoods such as hickory or maple are usually fed as chips or dust into the burner. Alternative processes include the use of approved distillates of natural smoke either in the brine solution or added to the cheese itself. The latter procedure is frequently used in processed cheese and occasionally with cheeses such as Mozzarella, which go through a melted curd stage. Utilization of this procedure ensures a smoked flavor throughout the entirety of the cheese, as well as a more uniform smoked flavor from day to day.

9. *Application of approved chemicals*

The most widely used chemical preservative in cheese is sorbic acid and its salts. It is a diolefinic acid, the chemical formula is $\text{CH}_3\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{COOH}$. Sorbates are particularly active against fungi and their inhibitory activity is enhanced by sodium chloride. Sorbates are applied as a slurry, or as a spray, or as dry powder on cheese surfaces to control fungi. Sorbate impregnated films are also used in some plants for wrapping cheeses. In cottage cheese, sorbate is added to the dressing to control fungal spoilage. In processed cheese, cheese spreads, and cheese foods, sorbates are added directly to the blended cheese in the cooker, blender, or mixer. Sorbates are sometimes misted or atomized on the surface of barre cheese just prior to closing the barrel. Likewise, a fine spray may be used on the surface of a number of other cheeses just prior to sealing the package. Occasionally an off-flavor, petroleum-like in nature, is associated with the inferior quality of sorbates or with their misuse.

In certain European countries, pimaricin, an antimycotic agent, is used.

Other chemicals which are permitted are of natural and processed cheese include lactic acetic, and citric acids, phosphates, citrates stabilizers, and sugar. These components may act by decreasing the pH or the A_w or by a direct effect on the growth of the bacteria, such as inhibition of streptococci and lactobacilli by high levels of phosphates.

10. Packaging

Packaging is a supplementary preservative principle. Ancient Romans recognized this and used clay to cover one of their cheese varieties, which possibly could have been the forerunner of present day Romano. The clay covering prevented dehydration and excessive rind formation. Olive oil is often rubbed on grana type Italian cheeses to prevent excessive moisture loss and hardening of rind. Use of cheese cloth as a bandage and for "dressing" is another form of protective packaging. Waxing also falls under this heading.

At present, clear or opaque heat-sealable films of various gaseous exchange coefficients are used. Usually, when plastic bags are used over the cheeses, a vacuum is drawn to eliminate the air, and the bagged cheeses are sprayed with or immersed in hot water to shrink the film and obtain a tight cling of the plastic material to the cheese surface. Aluminum foil is also used for varieties like Blue cheese.

Varieties of Cheese

1. Source of Milk - Mammalian Species Involved

Milk from different mammalian species vary in composition and flavor attributes. In different parts of the world, cheese is made from cow milk, buffalo milk, goat milk, milk from ewes, camels, reindeer, and yaks.

There are "growths" of milk just as there are "growths" of wine. The way the cows are fed, their breed, and weather conditions in the region can make a great difference in the quality of the milk that will be used in the cheese.

Buffalo milk is very high in butterfat content and lacks the yellow color of carotene. Also, because of higher total solids, buffalo

milk forms a firmer curd. Surti, a soft cheese made in India from buffalo milk has a firm body and is white.

Goat milk is characterized by its strong odor and flavor. The strong odor and flavor is contributed by the composition of goat milkfat, which contains a much higher level of C6 to C10 fatty acids as compared with cow milkfat. Caproic acid is especially "goaty" in odor. Goat milk also lacks the creamish-yellow color of carotene. Cheeses made from goat milk are whitish and have strong odor, flavor, and piquante (sharpness). A good example of such a cheese is Feta which is a Greek variety.

2. Kind of Milk Used – Treatment of Milk

Cheeses made from good quality raw milk have full intense flavor. Native enzymes in milk, especially the protease and the lipase have a role in flavor development during ripening. Cheese is also made from pasteurized milk. Pasteurized milk cheeses have a milder and more uniform flavor. Also flavor and body-texture defects associated with raw milk flora are not encountered in pasteurized milk cheese. From a public health point of view also, pasteurized milk is safer especially in the manufacture of semi-soft, high-moisture, mild flavored cheese like Camembert and Brie. Pasteurized milk as defined for cheese making refers to heating milk to 63°C and holding at the temperature for 30 min or any other time-temperature combination that would result in the inactivation of native alkaline phosphatase of milk. It is necessary that all unripened cheese be made from pasteurised milk.

Another milk treatment that is approved in cheesemaking is the use of hydrogen peroxide to destroy undesirable microorganisms.

Variation in cheese is also caused by the starting (or raw) material used in its manufacture, for example, if the cheese was made from whole milk, skimmilk or whey. Whole milk cheese are mellower, fuller, and richer in flavor than skimmilk cheeses. This is because milkfat mellows the tartness and sharpness of acid in cheese and provides fullness and richness to flavor not only through uniform distribution of milkfat *per se*, but also through flavorful compounds derived from breakdown of milkfat.

In manufacturing Cream cheese, 11% milkfat cream mix (containing optionally gums, salts etc.) is pasteurized at 69°C for 30

min, cooled to 49°C and homogenized single stage at 141.1kg per cm². Single-stage homogenization promotes slow drainage of whey and uniform distribution of fat globules, through mixing of the ingredients in the cream mix and good cohesion and smooth texture and body in the finished product.

Another milk treatment that is used in the manufacture of Blue cheese and to a limited extent in Mozzarella and Swiss cheese is decolorization or bleaching of milk. Sheep's milk is relatively whiter than cow's milk because there is very little carotene in ewe's milk. Roquefort cheese which is made of sheep's milk, therefore, has a whiter interior with deep blue veining. Cow's milk is creamy to light yellow depending upon the breed, and cheese made from cow's milk has a creamy-yellowish tinge to it. To approach the whiteness associated with Roquefort, cow's milk is bleached with benzoyl peroxide and other approved chemicals. Cow's milk intended for Blue cheese manufacture may be bleached using benzoyl peroxide or a mixture of benzoyl peroxide with potassium alum, calcium sulfate and magnesium carbonate. The weight of benzoyl peroxide should not be more than 0.002% of the weight of milk being bleached and the weight of the other ingredients allowed singly or combined and should not exceed 0.012% of the weight of milk being treated.

In Italy, Mozzarella originally was made from buffalo milk. Buffalo milk lacks carotene and hence is white in color. Therefore, a white satin-like shiny appearance is usually associated with Mozzarella cheese. To simulate the whiteness associated with imported Mozzarella. Benzoyl peroxide treatment of milk is allowed for Mozzarella manufacture in the U.S. The bleaching treatment is also allowed for other Italian cheese varieties like Parmesan, Provolone and Romano.

3. Microorganisms Added

Of the various factors involved in introducing differences between cheese varieties, microorganisms play a major role. Microorganisms added to cheese milk as starters or to cheese curd as supplementary starters and other microflora that comprise the "smear" are important in the formation of the characteristic body, texture, flavor, and color of various cheeses.

In certain cheeses, starter bacteria are used for curd formation through the production of lactic acid.

The choice of the bacterial species and relative rates of usage of starters are variable for different cheese varieties. Where the curd is cooked at higher temperatures (above 40°C) to squeeze out the moisture from the curd, the steady acid development throughout the cooking process is insured by using thermophilic and thermotolerant bacteria such as *Streptococcus thermophilus* and *Lactobacillus helveticus* or *Lactobacillus bulgaricus* or in some cases *Lactobacillus lactis*. In cheese cooked at moderate temperatures (maximum of 40°C) and in cheeses where moisture expulsion and firming of the curd is achieved with little or no elevation of temperature (held between 30 and 32°C), mesophilic starter bacteria are used - namely, the lactic streptococci comprising *Streptococcus lactis*, *Streptococcus lactis* subsp. *diacetylactis* and *Streptococcus cremores*. For certain cheeses *S. lactis* subsp. *diacetylactis* may be replaced by *Leuconostoc cremoris*.

4. Use of Enzymes

The most widely used enzyme in cheese making is calf rennet. Rennet is the crude mixture of enzymes extracted from the abomasum of a suckling calf. Rennet consists of a milk-clotting enzyme called chymosin and bovine pepsin, which has a strong proteolytic activity and a low pH optimum. Chymosin is an acid protease. In selecting and grading rennet preparations the major criterion used is the ratio of milk clotting to proteolytic activity. The higher the ratio, the higher is the grade. High proteolytic activity must be avoided. It lowers the yield of cheese, and during cheese ripening it may cause a weak body, bitterness, or other flavor defects. Besides clotting to proteolytic ratio, other characteristics considered are proteolytic specificity of the enzyme extract, characteristics of milk curd produced by the preparation, presence of contaminating enzymes and heterogeneity of proteinases in the extract. The milk coagulating mechanism of rennet consists of the cleavage of the macropeptide fraction within the V-shaped fold of kappa-casein. The release of the macropeptide destabilizes the kappa fraction and sensitizes casein micells to calcium ions. In the presence of free calcium ions, the colloidal suspension of casein collapses and clotting occurs. The presence of other enzyme fractions that can attack α and β -casein in rennet preparations is undesirable. Other contaminating enzymes like lipases are also detrimental to final flavor in cheese.

Because of the wide variety of rennets used in cheesemaking, there is variability in the response of commercial rennets to pH, temperature, and ionic concentrations. Also the retention of various enzyme preparations in the cheese curd differs; only 6% of the original activity of calf rennet is carried over in the cheese curd and about 35% of the original activity is destroyed during manufacture of Cheddar cheese. On the other hand, microbial rennets are stable to various manufacturing treatments in Cheddar cheese manufacture and about 2 to 3% of the original activity is retained in the pressed curd. Because these enzyme preparations contain various proteases from different sources, their activity against different protein components in milk also is variable.

5. Manufacturing Procedures

Manufacturing steps for various cheese varieties have developed over the years through trial and error. Cheesemaking in essence is the controlled expulsion of moisture from a milk curd. The extent of moisture removed from the curd varies with different cheeses. So also the methods used to achieve controlled expulsion of whey are different for different cheese varieties. Removal of moisture from the curd is primarily achieved through variations in mechanical handling of the curd, elevation of temperature during cooking, and promotion of acid development by starter bacteria.

Among the mechanical handling procedures, the cutting size of the curd is very important. Cutting has a two-fold effect one direct and the other indirect - on moisture release from the coagulated curd mass. When the coagulum is cut, a certain amount of whey entrapped between the snapped casein cross-strands escapes. This is the direct effect. The second, indirect effect sets the stage for further moisture expulsion during succeeding operations. By cutting the large mass of curd in the vat is subdivided into small pieces to create exposed surfaces from which whey can escape. The number of cuts, the distance between the cuts, and thus the size of the curd pieces has a direct bearing on the "effective surface area" for moisture expulsion. The smaller the pieces, the greater is the surface area and *vice versa*. Curd surface area is linked with external forces that influence moisture expulsion and if the surface area is greater the more intense are the effects of the external forces. The size of the curd also decides the distance trapped whey must work its way through the curd network to the exposed surface to escape.

So, by selecting the proper size for the knives to be used, the cheesemaker can control the amount of whey expelled during the manufacturing process.

In the manufacture of cheeses that are relatively sweet, and which require very little acid development during cheesemaking, whey expulsion is facilitated by cutting the coagulum into very small pieces, which then are cooked at a relatively high temperature (about 51°C). Cutting the curd into small pieces not only allows controlled expulsion of whey at low acidities, but also helps to obtain a tight knit of the curd which is necessary for evenly distributed eye development.

In Cheddar cheesemaking, the curd is cut with 0.64 cm knives and the size of the curd is not further reduced as in Swiss cheese manufacture. Moisture expulsion in Cheddar is achieved by promoting acid development and not by cutting the curd into *very* small cubes or by elevating the temperature as high as in Swiss cheese. The maximum cooking temperature for Cheddar cheese is 40°C.

Among the other curd handling procedures, adjustments in time-span and degree of stirring (gentle versus vigorous) of the curd cubes in the whey can introduce differences in cheese. Stirring curd in whey is believed to accelerate whey syneresis because of physical stresses on the curd. Stirring also facilitates keeping the curd cubes from coalescing or fusing into large lumps which reduces the effective surface area, and thus effective syneresis. Stirring should be gentle at the beginning of cooking when the curd is soft to prevent shattering and loss of cheese solids as fines in the whey. As the curd firms up stirring can be vigorous.

The method used for the separation of curd from whey is another item that contributes to differences in cheeses. The separation of curd and whey results in a partition of milk solids in the two phases. About 50% of the milk solids are lost in whey in Cheddar cheese manufacture. In the manufacture of Romano, the curd is separated from the whey by allowing the curd to settle to the bottom of the vat. In granular-type American cheese varieties, at the end of cooking operations the curd-whey mixture is pumped into a drain table where the whey is removed. In the case of Cream cheese, the whey is removed by centrifugation in a desludging-type separator. For the production of Baker's cheese and finely cut

Cottage cheese, whey may be separated from the curd by passing through a filter pad.

In the manufacture of Surti cheese, the rennet curd is spooned layer upon layer onto a small bamboo basket lined with cheese cloth and allowed to drain. The curd which fuses upon standing (about 30-32°C) is turned every 10-15 min to allow uniform draining. Salt is sprinkled upon every layer of curd before the next layer is spooned on. In small scale production of Blue cheese the curd is dipped onto a large cheese cloth suspended between a rectangular frame or a large rectangular perforated stainless steel cradle which can be placed across the top of the cheese vat and excess whey is drained off the curd. At this point, salt and mold spores may be added and uniformly mixed with the curd. The curd is then filled into hoops.

Salting is another process that contributes to differences between various cheese varieties. Cheese that are salted by immersing in brine, have an overall low salt concentration and the equilibration of salt concentration throughout the block takes a long time. Initially salt concentration is high in the exterior. Examples of such cheeses are Swiss cheese and Mozzarella. In varieties such as Blue cheese, the dry curd is first lightly salted, and additional salt is applied on the loaf either by dry salting or immersing in brine. In Blue cheese because of the high moisture and relatively open texture equilibration of the salt level is quicker.

Pressing hooped curd facilitates further expulsion of moisture from the cheese and in compressing individual curd pieces together into a tight knit. High moisture cheeses like Cottage, Camembert, and Blue cheeses are not pressed at all. Other semisoft cheeses are subjected to little or no pressure at all. Hard cheeses are pressed overnight with weights or by hydraulic pressure. Swiss cheese is pressed under whey to exclude air and care is exercised to obtain even pressure throughout especially at the edges and corners. A large amount of barrel Cheddar cheese is made at present. This cheese is not pressed. Barrels with suitable lining material are filled with milled, salted curd and as the curd settles in the barrel, the expressed whey is pulled out by vacuum suction.

Curing conditions also contribute to differences between various cheese varieties. Cheddar cheese and related varieties are cured between 2 and 7°C at a relative humidity not less than 50%.

After surface salting. Blue cheese loaves are pierced with needles to allow the diffusion of air for good blue veining. The cheeses are then placed in a room held at 10°C with a relative humidity of 98%.

In the production of Swiss cheese, after precooler holding, cheeses are transferred to a "warm room" held at 21 to 25°C to encourage active propionic acid fermentation. Periodically cheeses are sampled for eye-development and when the cheese core in the trier shows uniformly distributed eyes (about 4 eyes in the core evenly placed 2 to 3 cm apart), they are transferred to a finish cooler held at 2 to 5°C. The finish cooler holding arrests active propionic acid fermentation and firms up the cheese for easy handling, cutting etc. Also it prevents certain body and flavor defects.

Lastly, the shape and size of cheeses confer varietal status to certain cheeses.

Classification of Cheeses

At present, there is no single, universally accepted classification for cheeses. But a leading authority on cheese suggests the following criteria that could be used for classifying cheeses.

1. Natural versus processed
2. Type of starting material used namely, milk cream or whey
namely, milk cream or whey
3. Species of mammal
4. Cured versus uncured
5. Site of intensive cure, namely, surface or interior
6. Ripening agent(s), namely, bacteria and /or yeasts-molds
or enzymes
7. Country of origin
8. Methods of manufacture
9. General appearance
10. Physical or rheological condition
11. Chemical analysis

Table 5.1 : Classification of Natural Cheese

I. Soft Cheese (50 to 80% moisture)	<i>Ripened internally by bacterial fermentation</i>
<i>Unripened low fat</i>	<i>Pasta Filata</i>
Cottage	Provolone
Quark	Low moisture Mozzarella
Baker's	
<i>Unripened high fat</i>	III. Hard Cheese (Maximum 39% moisture)
Cream	<i>Internally ripened by bacterial fermentation</i>
Neufchatel	Cheddar
	Colby
<i>Unripened stretched curd or pasta filata cheese</i>	Caciocavallo
Mozzarella	<i>Internally ripened by bacterial fermentation plus CO₂ production resulting in holes or "eyes"</i>
Scamorze	Swiss (emmental)
<i>Ripened by external mold growth</i>	Gruyere
Camembert	Gouda
Brie	Edam
<i>Ripened by bacterial fermentation</i>	Samsoe
Kochkase	<i>Internally ripened by mold growth</i>
Handkase	Stilton
Caciotta (ewe or goat)	
<i>Salt-Cured or Pickled</i>	
Feta-Greek	IV. Very Hard Cheese (maximum 34% moisture)
Domiat-Egyptian	Asiago old
<i>Surface-Ripened</i>	Parmesan, Parmigiano, Grana
Liederkranz	Romano
	Sardo
II. Semi-Soft Cheese(39%-50% moisture)	
<i>Ripened by internal mold growth</i>	V. Whey Cheese
Blue	<i>Heat and acid denaturation of whey protein</i>
Gorgonzola	Ricotta (60% moisture)
Roquefort (sheep's milk)	<i>Condensing of whey by heat and water evaporation</i>
<i>Surface-ripened by bacteria and yeast (surface smear)</i>	Gjetost (goat milk whey; 13% moisture)
Limburger	Myost, Primost (13-18% moisture)
Brick	
Trappist	
port du Salut, St. Paulin	
Oka	VI. Spiced Cheese
<i>Ripened primarily by internal bacterial fermentation but may also have some surface growth</i>	Caraway-caraway seeds
Munster	Noekkelost-cumin, cloves
Bel Paese	Kuminost-cumin, caraway seeds
Tilsiter	Pepper-peppers
	Sapsago-hard grating, clover

Two broad divisions, however, are universally recognized. The first category would include *natural* cheeses and the second *processed* cheeses. Natural cheeses could be subdivided into those that are cured before consumption and those that are consumed fresh. Further groupings could be based upon the relative moisture contents of cheeses and the kind and distribution of microflora involved in their curing.

Natural cheeses. Natural cheeses are those that are consumed as prepared from milk or whey. In other words, these cheeses are consumed fresh or after ripening or curing without further treatment or processing.

Pasteurized process cheeses or processed cheeses. Processed cheeses are blends or combinations of one or more lots of one or more varieties of natural cheese subjected to comminuting, blending, heating, homogenization and in some instances, the addition of condiments, salt, water, cream, fruit, meats, and nuts.

Cured or ripened cheeses. These include the varieties that are held at relatively low temperatures under certain minimum levels of relative humidity (for most cheeses above 50-60% R.H.) for a minimum length of time to render them safe for consumption, and to induce specific changes in the body, texture, flavor, and color characteristic of the specific cheese variety.

Fresh, uncured or non-ripened cheeses. Cheeses in this category are those that are consumed without any ripening. These cheeses usually have a pleasant acid, "fresh dairy" (diacetyl) or bland flavor. The blandness of flavor, however, depends upon the species of animal from which the milk is obtained namely, cow or goat or sheep or mare or yak. Fresh, non-ripened cheeses are generally high in moisture and have a relatively short shelf life. Typical examples are Cottage and Feta cheeses.

There are nearly 400 varieties of natural cheeses, but based on physical and microbial characteristics, there are probably 18 or 19 basic types; others are variations of these basic types. A classification scheme is shown in Table 5.1.

Others has classified different cheeses as shown in Table 5.2.

Table 5.2 : Classification of Cheese by OLSON (1979)

-
- I. Natural Cheeses**
 - A. Cheeses varieties in which milk is clotted by acid:**
 - Cottage cheese
 - Baker's cheese
 - Cream cheese
 - Neufchatel cheese
 - B. Cheese varieties in which milk is clotted by Proteases:**
 - 1. Cheddar cheese
 - 2. Colby and Stirred curd (Granular) cheeses
 - 3. Surface-ripened cheeses-Brick cheese, Lumburger Cheese, Port du Salut, Bel Paese and Tilsit cheeses.
 - 4. Other Semi-soft cheeses-Edam, Gouda, Monterey and Munster cheeses.
 - 5. Cheeses with eyes-Swiss, Gruyere, Samsøe
 - 6. Italian Type
 - a) Very hard (Grating)- Parmesan and Romano
 - b) Other hard- Asiago, Fontina
 - c) Pasta Filata-Provolone, Mozzarella
 - 7. Mold-ripened
 - a) Blue, Roquefort
 - b) Cheeses with surface mold - Camembert, Brie and Coulommiers
 - II. Process Cheese**
 - 1. Processed Swiss, Processed Cheddar etc.
 - 2. Cold-Pack cheese
-

C. Process Cheese

Process cheese is food made from several lots of cheese that are comminuted or ground and mixed together by stirring and heating. Water, seasoning, color, and emulsifying salts may be added. Flavoring materials like pimentos and spices are sometimes used, or the cheese may be smoked or flavored with smoke condensate or precipitated smoke for additional character. Cheddar cheese is the variety most commonly processed in the United States. Process cheese is also made with swiss. Limburger, Brick and similar varieties. The finished product usually contains somewhat more moisture than the original cheese from which it was made.

Process cheeses have gained wide popularity because of the uniformity in quality, safety from a public health standpoint, longer keeping quality with or without refrigeration, and flexibility in merchandizing as small cuts, slices, shreds, as well as in grated form. Also, it allows canning of cheeses.

The main steps in the production of Process cheese consist of the following;

(1) Selection and cleaning of cheese: This consists of selecting natural cheese by lots giving consideration to intensity of flavor and body characteristics. Cleaning of cheese refers to surface scraping that may be necessary to remove mold spots or stains.

(2) Grinding or comminuting: In this process, the selected cheeses are cut up into small pieces, fed through grinders and mixers.

(3) Mixing: This may be a part of the grinding process in smaller operations. In large-scale production, mixing is a separate operation and is done in the cold. Salts are added during mixing.

(4) Cooking or pasteurization: Cooking is done in kettles or horizontal, lay-down cookers. In direct steam cooking, sanitary steam at 4.5-6.75 Kg Per 6.45 cm² is used. At the beginning of the cooking, visible fat appears. During the early stages some water may be added and additional salt is added to bring up the NaCl level to 3%. Along with salt, emulsifiers are added up to a level of 3%. Annato may also be added. At about 57°C the separated fat begins to emulsify and starts to disappear. At this stage, the cheese mix has the appearance of a rough (not smooth) but homogeneous curd. When the temperature reaches 66°C, the curd mass becomes plastic, fluid, smooth, and silky. The temperature is raised to 71°C with stirring and held for 3 min. During this period, adjustment of moisture level and pH is done.

(5) Filling (or packaging) and cooling: The cheese is packaged while still hot and is piped to the filling machines. Packaged cheese is slowly cooled by rolling the packages into coolers held at 20-21°C. As the cheese cools, it gets firmer. After the packages have been cooled to 20-21°C, they are transferred to coolers held at 7°C, where the cheese firms up further as the fat solidifies.

Club or Cold Pack cheeses: Club cheese may be considered under the heading "Process cheese" because these cheeses cannot be

Table 5.3 : Processed Cheeses

<i>Category</i>	<i>Ingredients Allowed</i>	<i>Cook Temp.</i>	<i>% Water/% Fat</i>	<i>pH</i>
Processed Cheese	Cheese, color, salt, emulsifier (3%), smoke, spices	68-71°C	Variable according to cheese variety	5.6-5.8 > 5.3
Processed Cheese Food	Same as in Processed cheese, but optional ingredients like skim milk, whey, cream, albumin, skim milk cheese, organic acids	77-82°C	Water not > 44% Fat not < 23%	5.2-5.6 > 5.0
Processed Cheese Spread	Same as in Processed Cheese Foods, but gums may be added to hold moisture and for binding. Sugar(s) also allowed	85-88°C	Water not < 44% or > 66% Fat not < 20%	5.2 or lower but > 4.0
Club or Cold Pack Cheese	Cheese, color, water acid(s)	Not cooked	Same as for natural cheese standards	> 4.5

categorized as natural cheese. Although no cooking or pasteurization is involved in the production of Cold Pack cheese, addition of condiments, wine, smoke flavor, or other flavorings is allowed.

In the production of Cold Pack cheeses, natural cheeses that are either made from fully pasteurized milk or which have been cured for at least 60 days are selected for varying degrees of sharpness and ground together and blended with flavorings etc. The blended cheese mix is fused together in molds and chilled. The product may be packed in porcelain crocks or in moisture proof materials.

D. Imitation Cheese

The advent of imitation cheese came about in response to two primary pressures. The more important of these related to economic considerations. The price of milk fat, a major cheese component, has always been higher than that of vegetable or seed oils. The second motivating factor was the "perceived health benefit" derived from deletion of cholesterol contributed by milk fat and lower saturated fatty acid-content in most vegetable fats which are wholly or partially substituted for milk fat in imitation cheeses.

A typical formula for imitation cheese would include caseinates (usually calcium caseinate), vegetable oil, emulsifiers, stabilizers, salt, acidulants, sodium citrate, flavors, color, and water. To retain an authentic cheese flavor, several modifications are used. In some cases, a combination of milk fat and vegetable fat is used. Others include some real cheese in the formula. Application of enzyme modification of basic ingredients is also used.

There is very little biotechnology involved in the production of most imitation cheeses, but basic research in food systems is needed in blending the right components in the proper order under exacting conditions. Currently the thrust is towards substituting a variety of proteins like wheat, soybean and peanut proteins for casein as the main functional ingredient. Other efforts are directed at:

(a) Developing flavor combinations that would be uniform and simulate "natural cheese flavor"

(b) Improving product functionality by using different stabilizer-emulsifier combinations such as sodium lactate, monoglycerides, and modified gums.

Cheese - A Dynamic Biological System

Biological Entities in Cheese Systems

Cheese is a unique biological system. Production of cheese involves the conversion of a biological fluid, milk, through the use of biological catalysts consisting of free enzymes in the form of native milk enzymes, rennet, and other glandular enzymes from mammals which are added to milk and the application of biochemical catalytic generators in the form of starter bacteria and associated yeasts and molds. Cheese is chemically, enzymatically and microbiologically a very complex and dynamic system.

Milk

As a biological fluid, milk is unique. Although milk is a liquid, it contains greater amounts of solids than many other food materials that occur in nature in solid form -e.g., fruits and vegetables. This unique feature of milk is made possible by well-known physico-chemical forces in nature. By counteracting some of these natural forces, one is able to convert fluid milk into a wide variety of dairy products including cheese.

The major component of milk is water which accounts for approximately 86-87% of the total weight. Water is the vehicle in which the various solid components of milk are dispersed.

From a technologist's point of view, it is the manner in which various components of milk are distributed that is of prime importance. A large number of the technological operations concerned with the manufacture of cheese involve partial removal of the water in milk, in the form of whey. The degree of moisture removal however, varies with the cheese variety.

The different solid components of milk are milk fats, milk proteins, lactose and minerals or ash. Milk fat or butterfat occurs in the form of minute globules distributed throughout the body of the lacteal fluid. Milk fat globules are enclosed in a membrane made up of phospholipid-protein. The individual fat globules are held apart from one another by the intervening water medium in which they are suspended. This sort of dispersion is referred to as an emulsion. Milk fat has the lowest specific gravity among the various milk components. Because of this, in whole milk, the milk fat globules migrate to the uppermost layer in the fluid to form the "cream line".

Lactose is the major sugar in milk. Milk also contains two or three minor sugars and sugar phosphates in trace amounts but these are of little consequence in cheese making.

Calcium and magnesium are other ash components that have relevance to cheese technology. Calcium is necessary for good rennet coagulation. The relative proportions of calcium and magnesium in dissolved and undissolved states in milk and the equilibrium between these phases are usually referred to as the "mineral balance" of milk. The mineral balance affects the relative stability of the casein colloidal system when heat is applied, the strength of the curd formed during cheese making, and the rheology (plastic characteristics) of the cheese curd.

Among the minor milk constituents, vitamins and enzymes are directly or indirectly involved in cheese technology. Vitamins as such, apart from a nutritional role for starter microorganisms and for the consumer, do not have a functional role in cheese technology. Vitamin A in cow's milk occurs as carotene (a precursor of retinol), a pale yellow pigment that imparts a creamy, pale yellow color to milk. Carotene is fat-soluble. In the manufacture of certain cheese varieties, where an absolute white color is desired - e.g., Blue cheese, Mozzarella etc. carotene is destroyed by the use of bleaching agents like benzoyl peroxide. When milk or cream is bleached for cheese-making vitamin A in the form of a palmitate is added to restore the precursor destroyed by bleaching.

2. Microorganisms

Microorganisms involved in cheese manufacture and curing may be conveniently divided into two groups, namely, starter flora and adventitious flora.

Starter Bacteria

Starter bacteria as defined in cheesemaking refer to carefully selected microorganisms that are *deliberately* added to milk or cream or a mixture of both to *initiate* and *carry through* the desired cheese fermentation. In other words, starters are selected strains of microorganisms intentionally added to milk or cream or a mixture of both during conversion into cheese to bring about specific changes in the appearance, body, texture, and flavor characteristic of the desired final product.

Table 5.4 : Starter and Associated Flora of Major Cheese Varieties

<i>Cheese Type Flora</i>	<i>Name of Cheese</i>	<i>Cured or Uncured</i>	<i>Starter Species</i>	<i>Starter Function</i>	<i>Associated Flora</i>	<i>Function of Associated</i>
High moisture (80%), soft	Cottage Cream Neufchatel	Uncured	<i>Streptococcus lactis</i> <i>S. cremoris</i> <i>Leuconostoc cremoris</i>	Acid production Flavor production	<i>S. lactis</i> subsp. <i>diacetylactis</i> in Cottage cheese cream dressing	flavor production Increase shelf life
High moisture (80%), soft	Camembert Brie Coulommiers	Cured mainly surface	<i>S. lactis</i> <i>S. cremoris</i> <i>S. lactis</i> subsp. <i>diacetylactis</i>	Acid production Some flavor production	<i>Penicillium camemberti</i> <i>P. caseicolum</i> <i>Brevibacterium linens</i> <i>Micrococcus</i> spp. yeasts	Surface curing and flavor production
Semi-soft moisture 50%	Brick Edam Gouda	Cured internal and surface	<i>S. lactis</i> <i>S. cremoris</i> <i>S. lactis</i> subsp. <i>diacetylactis</i>	Acid production Flavor production Gas openings in Dutch cheese	<i>Brevibacterium linens</i> <i>Micrococcus</i> spp. yeasts	Light to medium surface-curing flavor production
Semi-soft moisture 50%	Limburger Trappist	Cured internal and surface	<i>S. lactis</i> <i>S. cremoris</i>	Acid production Some flavor production	<i>Brevibacterium linens</i> <i>Micrococcus</i> spp. yeasts	Extensive surface curing flavor production

contd...

Table 5.4 – contd...

<i>Cheese Type Flora</i>	<i>Name of Cheese</i>	<i>Cured or Uncured</i>	<i>Starter Species</i>	<i>Starter Function</i>	<i>Associated Flora</i>	<i>Function of Associated</i>
Semi-soft moisture 50%	Roquefort Blue	Cured internal mainly	<i>S. lactis</i> <i>S. cremoris</i> <i>S. lactis</i> subsp. <i>diacetylactis</i> <i>Leuconostoc cremoris</i>	Acid production Promote gas openings	<i>P. roqueforti</i> Smear flora	Flavor production Blue veining
Hard, moisture 36-39%	Cheddar stirred curd	Cured internal only	<i>S. cremoris</i> a few <i>S. lactis</i> strains	Acid and flavor production	None	None
Hard, moisture 39%	Italian Pasta Filata types	Cured internal only	<i>S. thermophilus</i> <i>Lactobacillus</i> <i>helveticus</i> <i>L. bulgaricus</i>	Acid and some flavor production	None	None
Hard, moisture 39%	Swiss cheese varieties	Cured internal only	<i>Streptococcus lactis</i> <i>S. cremoris</i> <i>S. thermophilus</i> <i>L. helveticus</i> <i>L. bulgaricus</i>	Acid and some flavor esp. lactobacilli	<i>Propionibacterium</i> <i>freudenreichii</i> and <i>P. freudenreichii</i> subsp. <i>shermanii</i> Smear flora in Gruyere	Formation of eyes and characteristic flavor. Flavor from light smear flora in Gruyere
Very hard, moisture 34%	Italian Grana type	Cured internal only	<i>S. Thermophilus</i> <i>L. helveticus</i> <i>L. bulgaricus</i>	Acid and flavor	None	None

Other specific functions of starter cultures mainly relate to flavor development. The role of the starter culture in cheese flavor development is either direct or indirect. The direct contribution is due to compounds such as lactic, acetic, and propionic acids formed by the metabolism of lactose. Acetic and propionic acids are important in Swiss cheese flavor. Additionally, carbohydrate metabolism by starter bacteria also yields small amounts of odoriferous and flavorful compounds such as aldehydes (especially

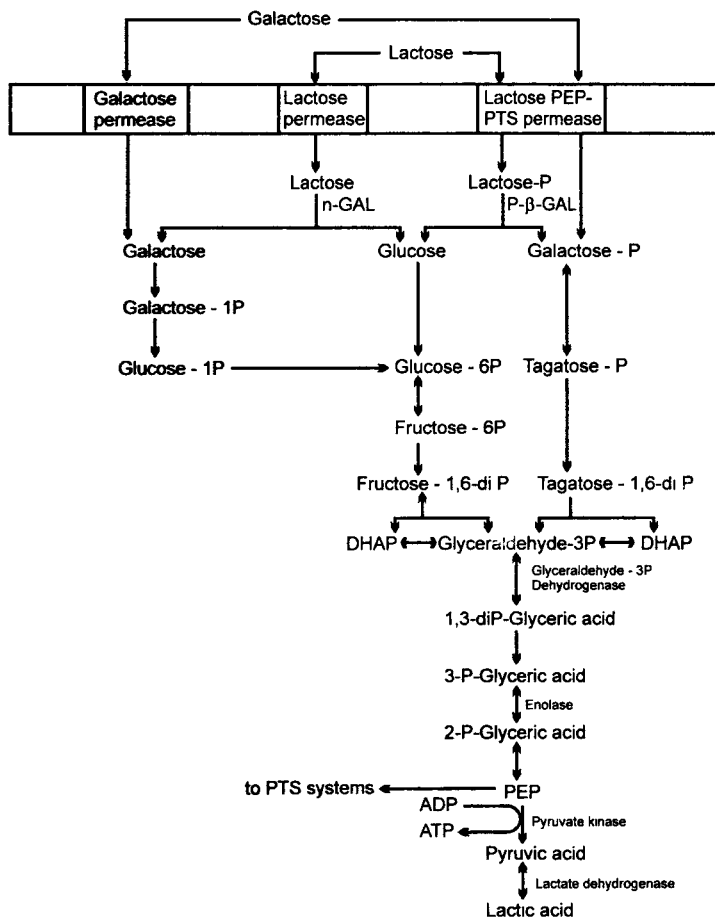


Fig. 5.1 : Pathways for lactose and galactose catabolism in dairy streptococci. – A strain may possess one or more of the indicated transport mechanisms.

acetaldehyde) and ketones (acetone and diacetyl) or their precursors such as pyruvic acid and other volatile compounds like ethyl alcohol and volatile fatty acids which react to form fatty esters like ethyl acetate, propionyl acetate etc., which are important in the flavor profile of cured cheeses.

In Table 5.4 the various starter bacteria and other supplementary flora involved in the manufacture of different cheese varieties are listed.

In milk, where the major carbon and energy source is lactose, the fermentation is facilitated by specific transport mechanisms that translocate (Fig. 5.1) the sugar from the exterior into the interior of the cell membrane, where it is cleaved into component monosaccharides and fed into aforementioned specific pathways after necessary modifications. The mechanisms of lactose utilization and the genetic determinants that govern the function in starter bacteria have been the subject of intensive research over the past decade and a half. These findings are summarized in Table 5.5.

A summary of the enzymatic mechanisms for citrate utilization by flavor bacteria is given in Table 5.6.

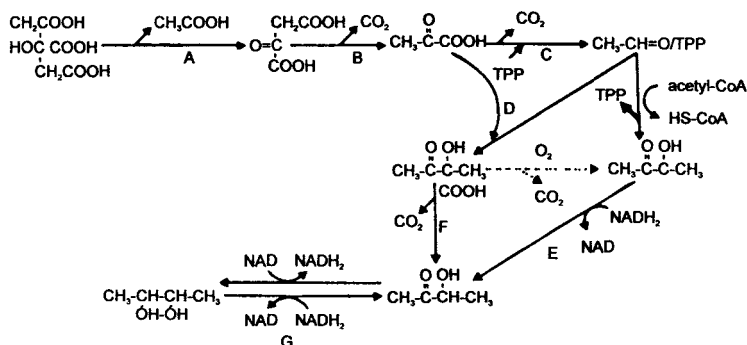


Fig. 5.2 : Biosynthetic pathway among dairy lactic acid streptococci for the production of diacetyl and its reduction products from citric acid.- A citratase; B oxaloacetate decarboxylase; C pyruvate decarboxylase; D α-acetate synthetase; E diacetyl reductase; F α-acetyl-CoA decarboxylase; G 2,3-butanediol dehydrogenase. Broken line represents the step on which disagreement exists in the literature. It is thought of either as a non-enzymatic reaction or an enzymatic step catalyzed by α-acetolactate oxidase.

Table 5.5 : Mechanisms for Lactose Utilization by Starter Bacteria

<i>Starter Bacteria</i>	<i>Lactose Transport System(s)</i>	<i>Lactose Cleavage Enzyme</i>	<i>Genetic Determinant for Lactose Utilization</i>	<i>Major Carbohydrate Metabolic Pathway</i>	<i>Major End Products</i>
<i>Streptococcus cremoris</i>	PEP-phosphotransferase	Phospho- β -galactoside galactohydrolase (P- β -gal)	Plasmid borne	HDP	Lactic acid
<i>Streptococcus lactis</i>	PEP-phosphotransferase	P- β -gal	Plasmid borne	HDP	Lactic acid
<i>Streptococcus lactis</i> subsp. <i>diacetylactis</i>	PEP-phosphotransferase	P- β -gal	Plasmid borne	PK	Lactic acid, acetic acid, and CO ₂
<i>Streptococcus thermophilus</i>	β -galactoside permease	β -galactosidase (β -gal)	—	HDP	Lactic acid
<i>Homofermentative lactobacilli</i>	Either β -galactoside permease or PEP-phosphotransferase or a combination of both	P- β -gal or combination of both	Plasmid borne in some species	HDP	Lactic acid
<i>Leuconostoc cremoris</i>	Not known	Not known	Not known	PK	Lactic acid, acetic acid, CO ₂ and ethanol

Table 5.6 : Enzymatic Mechanisms for Citrate Utilization by Flavor Bacteria in Starters

<i>Species</i>	<i>Citrate Transport Enzyme (CT)</i>	<i>Genetic Determinant for CT</i>	<i>Mode of CT Expression</i>	<i>Citrate Cleaving Enzyme (CC)</i>	<i>Genetic Determinant For CC</i>	<i>Mode of CC Expression</i>
<i>Streptococcus lactis</i> subsp. <i>diacetylactis</i>	Citrate permease active below pH 6.0	5.5 Mdal plasmid	Inducible	Citratase	Chromosome	Constitutive
<i>Leuconostoc cremoris</i>	Citrate permease active below pH 6.0	Not known	Inducible	Citratase	Chromosome	Inducible

In addition to the aforementioned indirect relationship of starter proteolytic function to cheesemaking, slight proteolytic activity by starter bacteria is necessary during curing to obtain mellowness and suppleness of body and typical cheese flavor.

Bacteriophages

Bacteriophages, as one of the biological entities involved in cheesemaking play a negativbe role. Bacteriophages are viruses of bacteria, and are commonly referred to as *phages* in the literature. Phages are tadpole-shaped submicroscopic particles with a definite head and a long tail. The head and the tail are made up of protein. The head encloses the phage nucleic acid and the tail a hollow space connected to the interior of the head. The tail is striated and contractile. The tail may have a tail plate and spikes attached to the plate. Phages exist in different functional states.

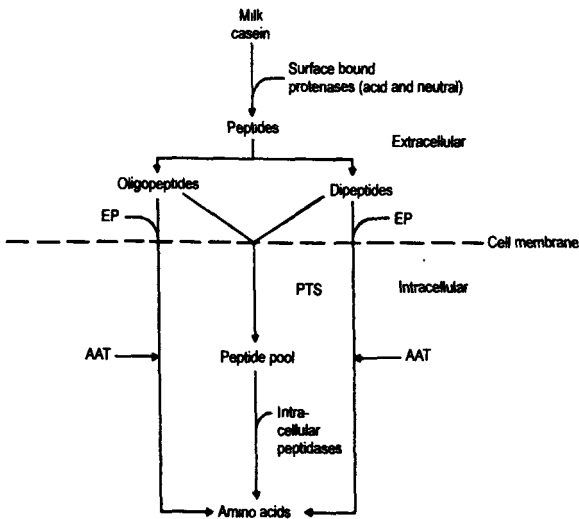


Fig. 5.3 : Proteolytic systems of starter bacteria. - AAT amino acid transport system; EP extracellular free and bound peptidases; PTS peptide transport systems.

Intracellular proteinases are liberated when cells lyse in the cheese system. They contribute to protein breakdown at that stage.

Cheese Curing and Cheese Flavor

Cheese curing is the process by which the raw, green, chewy, rubbery bland curd after certain post-hoop treatments is held under controlled conditions of temperature and humidity for a sufficient length of time and is transformed into mellow, waxy, and flexible substance that acquires the color, body, texture, and flavor characteristic of the cheese.

Cheese Production

Milk Quality and Composition

The first step in the production of good cheese is the production or acquisition of good milk. The quality of raw milk can be described principally under five headings.

1. Normal milk constituents
2. Number and type of microflora
3. Leucocyte or somatic cell count
4. Degree of adulteration
5. Miscellaneous factors

Normal Milk Constituents

Factors affecting the compositional make-up of normal milk may be divided into those that are genetic and those that are non-genetic. The genetic factors include differences between individual animals as well as those among breeds and species. The non-genetic factors include stage of lactation, type of feed, environmental temperature and humidity, age of the animal, milk parlor techniques, etc.

Generally, all of the varieties of cheese contain the same milk components, but they vary in their concentrations depending on the composition of the original milk, the make procedure, and the type of fermentation that has occurred. Although most of the world's cheese is made from cow's milk, the milks of goats, sheep, camels, reindeer, water buffaloes and mares are also used. A comparison of the composition of milk from various species has been compiled from various sources and presented in Table 5.7.

Table 5.7 : Composition of Milk from Various Mammals

<i>Mammal</i>	<i>Protein</i>	<i>Casein</i>	<i>Proximal Analysis (%)</i>			<i>Total Solids</i>
			<i>Fat</i>	<i>Lactose</i>	<i>Ash</i>	
Cow	3.40	2.82	3.72	4.90	0.72	12.74
Goat	3.20	2.60	3.90	4.50	0.80	12.40
Mare	2.49	1.33	1.59	5.90	0.40	10.38
Water Buffalo	4.00	—	7.98	5.18	0.79	17.95
Sow	6.00	2.31	6.85	4.90	0.95	18.70
Camel	3.50	—	3.50	5.00	0.70	12.70
Reindeer	11.46	8.69	16.90	2.75	1.43	32.54
Sheep	6.00	4.80	7.00	4.50	0.90	19.00
Dog	7.10	—	8.30	3.70	1.30	20.40
Human	1.30	0.50	3.70	7.00	0.20	12.20

Table 5.8 : Proximal Composition of Milk from Six Dairy Cattle Breeds

<i>Component</i>	<i>Holstein</i>	<i>Jersey</i>	<i>Guernsey</i>	<i>Ayr-shire</i>	<i>Brown Swiss</i>	<i>Milking Shorthorn</i>
Crude Protein	3.22	4.22	3.70	3.47	4.05	3.42
True Protein	3.07	4.07	3.56	3.30	3.84	3.17
Casein	2.53	3.39	2.88	2.73	3.14	2.56
True Whey Protein	0.54	0.68	0.68	0.57	0.69	0.60
Fat	3.73	5.42	4.76	4.12	4.28	3.58
Lactose	4.93	4.99	4.66	4.67	5.15	4.80

It is evident from the data presented that cheese made from different mammalian milks may be markedly dissimilar. At though the compositional variations between breeds is not as great as those between species there are some significant differences.

Number and type of microflora

Raw milk withdrawn through the teat cistern of a healthy animal is extremely low in bacterial count. The temperature of milk that is picked up from the farm and delivered to the cheese plant should be less than 8°C. Proper handling and refrigeration should result in additional cooling to about 4°C in the milk holding silos. The further the milk temperature is depressed, the greater the control of microbial and enzymatic deterioration of milk.

Leucocyte Count

The composition of milk varies considerably with the type and severity of various pathological and subclinical conditions of the mammary tissue.

Degree of Adulteration

There are number of ways milk can be adulterated. Probably the most common type of adulteration is the addition of water - either intentionally or unintentionally. The addition of water to milk is detected by determining the freezing point, which averages minus 0.54°C for normal milk. The greater the amount of water added to milk the closer the freezing point will approach that of water.

Milk Components and Cheese Production

The components of natural cheese are primarily components of the milk from which it was produced. Other ingredients which may be added to the milk or to the curd are salt, cultures, culture media, calcium chloride, rennet and other enzymes, annatto, and potassium sorbate.

The physical and chemical changes which occur i milk components during the transformation of milk into cheese are limited to water, proteins, lactose, milk fat, and salts.

1. Water

a) In milk

Water is the major component of the milk from all mammals. Although a small portion of the water is bound to the lactose, protein, and salts, most of it exists in the free state and serves as the medium in which all other milk components are either dissolved or suspended. The significance of water in promoting and controlling innumerable physical and chemical reactions in milk such as the electrostatic and hydrogen bonding between enzymes and milk constituents and the functional folding of macromolecules has not been clearly elucidated.

b) In cheese

Conventional manufacture of most types of cheese requires the formation of an acid or rennet coagulum. This coagulum occupies the entire space that was once occupied by fluid milk. Subsequent

cutting of this coagulum into cubes results in the presence of two phases: The whey, a yellowish liquid, and the curd, which is the mass of semi-solid white cubes containing about 75-80% moisture. The objective from that point in manufacture until the packaging of the finished product is to bring about controlled expulsion of water from the curd portion until a previously specified target range is achieved.

2. Milk fat

a) In milk

The fat portion of milk is a major determining factor in the classification of cheese. This is the first milk component to be considered during the conversion of milk to cheese. Optimization of the fat content in milk by either separating out part of the fat or by adding extra fat is usually termed standardization. The point to which milk is standardized is determined by the variety of cheese one wishes to make, the percent solids-not-fat in the milk, and the amount of fat expected to be lost in the whey as determined by previous experience.

Milk fat is the most variable major milk constituent. Under normal conditions about 95 to 96% of the total milk lipid materials is triglycerides and another 1.5% is diglycerides. The predominant fatty acids in the glycerides, making up 75 to 80% of the total fatty acids in milk, are: myristic, palmitic, stearic, and oleic. Milk fat also contains vitamins A, D, E, and K, and various carotinoids. A small proportion of free fatty acids is found in fresh milk. As milk fat is hydrolyzed by either milk lipase or microbial lipase, the concentration of free fatty acids increases dramatically.

Milk fat exists as an emulsion in milk. It is a discontinuous dispersion in the water phase. Milk fat occurs in raw milk in the form of fat globules which are generally 2 to 6 microns in diameter. These fat globules are formed within the secretory cells of the lactating mammary gland when secretory vesicles containing lipid material are transported to the interior surface of the apical plasma membrane.

Average values of some additional characteristics of milk fat which are useful in the biochemistry of cheese are:

Saponification number	230
Iodine number	27
Reichert-Meissl number	28
Polenske number	2
Specific gravity	0.9300
Refractive index	1.4547

b) In cheese

Milk fat is a major functional component of most cheese, influencing the color and body as well as the flavor. Much of the color effects actually come from fat soluble materials such as β -carotene.

Milk fat in cheese is entrapped within the protein matrix unlike in fluid milk where it is in an emulsion. Fat hydrolysis in cheese is generally traceable to any one or combinations of four groups of lipases that may be encountered in cheese system. First is the lipase which occurs naturally in raw milk. Since this enzyme is almost completely destroyed by pasteurization (63°C for 30 min) of the milk, fat hydrolysis by milk lipase in cheese made from fully pasteurized milk is very low to undetectable. However, in cheese made from raw or subpasteurized milk action of milk lipase on milk fat is likely to occur.

The second group of lipases are those which originate from the microflora of the raw milk. These microbes are usually killed by heat treatment of the milk or they die off during cheesemaking and the subsequent curing process.

The third group of lipases are those that are part of the enzymatic make-up of supplementary starter. The best example is the lipolytic system of *Penicillium roqueforti* which is an integral part of Blue, Roquefort, Gorgonzola, and Stilton cheeses.

The fourth group of lipases are those which are added as non-living entities to either the milk or the curd. These lipases are commercially available usually as a paste made from grinding the section of tissue from the base of the tongue to the first epiglottal ring of the young goat.

3. Lactose

a) In milk

Lactose is synthesized in the mammary tissue, primarily from blood glucose. Alpha-lactalbumin serves as a component of the synthetase system. In fluid milk, lactose occurs totally in solution. Its main function is as an energy source for young mammals. In addition, lactose contributes some sweetness to the milk. When considered strictly from the standpoint of cheese production the lactose in milk is functional only as a substrate for microbial fermentation, predominately to lactic acid.

b) In cheese

The presence of lactose in the vat coagulum and in the subsequent cheese is a result of mechanical entrapment of whey in the micellar matrix. The direct influence of lactose is limited to events occurring during cheese manufacture and the early stages of ripening.

4. Protein

a) In milk

The protein in natural cheese comes from milk. The type of protein and, to some degree, the concentration depends on the cheese manufacturing procedures. Although the cheesemaker generally limits his distinction of milk proteins to two simple classes, i.e. casein and whey protein, there are actually six classes of proteins. These are presented in Table 5.9 along with their approximate concentrations in milk. Casein in fluid milk occurs in the form of colloiddally suspended micelles. There are approximately 10^{12} micelles per mL.

Casein is a dominant milk component governing the yield of cheese that can be derived from a given quantity of milk. Most formulae for determining cheese yield utilize the percent casein in the raw milk. However, a rapid, accurate quantitative test for casein is presently not available. Therefore, total protein is determined and 78-80% of this value is assumed to be casein.

b) In cheese

The major protein component in all milk cheeses is casein. In whey cheese there is very little casein and the proteins consist

Table 5.9 Approximate Concentration of Six Major Protein Constituents In Cow's Milk

<i>Protein</i>	<i>Concentration in Milk (%)</i>	<i>Total Milk Protein (%)</i>
Total caseins	2.62	79.40
α -Casein		45.00
κ -Casein		8.00
β -Casein		25.00
γ -Casein		3.00
β -Lactoglobulin	0.25	7.58
α -Lactoglobulin	0.15	4.55
protease peptone	0.15	4.55
Immunoglobulins	0.10	3.00
IgG		2.00
IgM		0.40
IgA		0.10
Blood serum albumin	0.03	0.92
Total protein	3.30	100.00

primarily of lactalbumin and lactoglobulin. The protein, especially casein, is found in a continuous phase in cheese as opposed to occurring as a discontinuous (colloidal) phase in fluid milk. The role of protein in determining the attributes of each variety of cheese is only partially understood. Electron micrographs, as well as other analytical data, have demonstrated the development of a cross-linked, fibrous protein matrix during the transformation of milk into coagulum and the coagulum into cheese.

5. Salts and Minerals

a) In milk

Minerals play an extremely important role in cheese milk. This component includes both the non-metallic elements, such as phosphorus, and the metallic elements, like calcium and magnesium. Minerals are found in both the dissolved or aqueous phase (whey) and in the discontinuous (colloidal) phase of milk. The equilibrium of these minerals, as well as their salts and acid radicals, depend on the ionic concentration, temperature, pH, solubility of each constituent, and the dissociation constants.

Calcium, the mineral most frequently associated with milk, occurs in three forms and, therefore, presents a very complex system. About 10% of the approximate total of 125 mg per 100 g is

in ionic form and about 20% is complexed with casein. The remainder of the calcium occurs primarily as soluble and colloidal salts of anions such as phosphate and citrate. Colloidal calcium phosphate (CCP) plays a major role, along with κ -casein, in the stability and integrity of casein micelles. CCP exists as amorphous tertiary calcium phosphate, which is only slightly soluble at the normal pH of milk. Some researchers suggest that half of the CCP is in the form of a highly insoluble hydroxyapatite which is prevented from precipitating by its association with casein micelles.

When heated milk is cooled it becomes unsaturated with regard to phosphate and calcium. If after heating, milk is left undisturbed the mineral content in the dissolved state will increase and again equilibrate between the dissolved and the colloidal states within 24 to 48 hours. These relationships in the milk salts should be kept in mind by the cheesemaker as the cheese milk is subjected to any or all of these treatments.

If during some seasons of the year or as a result of the milk treatment the calcium balance is disturbed, calcium chloride, dibasic calcium phosphate, calcium lactate, or calcium hydroxide may be used to restore the calcium and optimize the coagulation time. Calcium chloride addition to cheese milk is a widely accepted practice and has been shown to reduce rennet coagulation time. This beneficial effect is not always observed and, in fact, some cheesemakers have reported negative effects resulting from calcium chloride usage. Evidence indicates that a low concentration of calcium is required for rennet coagulation. However, calcium concentrations above perhaps 50 mM actually prolong the coagulation time and decrease the firmness of the curd.

As previously stated, the equilibrium between the colloidal and dissolved states of calcium and phosphorus is not dependent solely on pH. Time, temperature, and ionic concentrations influence the concentrations of colloidal and free calcium and phosphate. These, in turn, affect the clotting time of milk. Therefore, milk with a titrable acidity of 0.25% coagulates at about 82°C. Likewise milk at 3°C may be adjusted to pH 4.6 with phosphoric acid without causing precipitation. Normal cow's milk may contain 0.12% calcium and maximum clotting occurs at 0.14%. Addition of too much calcium chloride may cause excessive dissociation of α_s - and κ -casein in the micelles and result in an inflexible, bitter curd. Too little calcium chloride also causes a brittle curd and prolongs

coagulation time. Fluid milk becomes more sensitive to heat destabilization when calcium chloride is added because the concentration of ionic calcium is raised. Although calcium addition may improve coagulation in many instances, it is certainly a wasted additive in some of its present usages.

The salt balance in milk is important in both acid and enzymatic coagulation. The secondary phase of coagulation unlike the primary phase, is highly dependent on calcium. The primary phase of rennet coagulation involves the cleaving of a peptide from the κ -casein entities in the micelles. In normal milk, about 80 to 90% of the κ -casein is altered before clotting is evident. However, no coagulation is evident below 10°C or above 65°C. Neither is coagulation realized in completely decalcified milk. An increase in calcium ion concentration decreases the clotting time as well as increases the curd strength. Phosphates in the colloidal state are also essential for a smooth clot. In some cases sodium phosphate is added to milk to restore the calcium ; phosphate ratio.

As the cheese curds are agitated in the whey, lactic acid bacteria mediate a decrease in pH as they ferment lactose to lactic acid. The calcium phosphate is progressively solubilized and leached out of the curd in the expelled whey. The acid whey from cheese, such as Cottage, contains much more calcium, manganese, zinc, and iron than does the sweet whey from cheeses such as Swiss. This means Swiss cheese contains more of the minerals than do acid cheeses. (See Table 5.10).

Table 5.10 : Typical Values for 5 Predominant Minerals in Several Common Cheese Varieties

Cheese Variety	Mineral Content (mg/100 g)				
	Pota- ssium	Calcium	Phos- phorus	Sodium	Magnesium
Blue	325	525	395	1230	23
Cheddar, mild	145	750	520	650	26
Colby	150	675	470	640	26
Cottage, creamed	115	65	155	410	6
Cream	130	70	110	280	7
Mozzarella, low moisture	290	740	485	580	27
Provolone	160	790	530	920	27
Ricotta, part skim	120	250	180	90	13
Swiss	120	1000	680	270	36

Table 5.11 : General Steps in the Manufacture of Cheese

-
1. Heat treatment of the milk
 2. Ripening (vat)
 3. Coagulation or setting
 4. Cutting
 5. Healing or contracting
 6. Foreworking
 7. Pre-drawing
 8. Cooking
 9. Stirring out
 10. Washing
 11. Draining
 12. Matting
 13. "Cheddaring"
 14. Milling
 15. Mixing-molding
 16. Hooping
 17. Pressing
 18. Salting
 19. Curing
-

Steps in the Manufacture of Cheese

Cheese manufacture comprises several distinct steps that could be considered individually for their distinct contribution to the process. These are listed in Table 5.11.

1. Heat Treatment

Milk used in the manufacture of most cheeses today undergoes some type of heat treatment. This treatment may be sub-pasteurization or full pasteurization and it may be accomplished in a processing vat or by HTST treatment. Some cheese manufacturers use an elevated temperature or an extended heating time to promote whey protein denaturation or complexing with casein in an

effort to increase cheese yields. Unless make procedure compensations are made to accomodate such special heat treatments, the quality of the cheese is affected.

2. Ripening

After a portion or all of the milk has been pumped to the vat and the temperature has been adjusted to the desired range, a quantity of starter culture is added. The culture may be a frozen concentrate, a lyophilized powder or a liquid bulk culture prepared by growing the starter organisms in milk, whey, or a commercial starter medium.

3. Coagulation

Since a primary objective in cheesemaking is removal of moisture from milk, it must be transformed to a physical state which lends itself to easy dehydration. Efficient dehydration of most food

items is promoted by first decreasing their physical size: for example, fruits and vegetables are sliced or chopped into smaller pieces to provide greater surface area for evaporation. To achieve this, milk must first be transformed to a solid or semi-solid state. This transformation is mediated by a coagulant or by acid or by a combination of the two. The rate of coagulation depends on the temperature, pH, milk composition, calcium, availability, heat treatment of the milk, and the type and amount of coagulant.

4. Cutting

The coagulated mass is cut into cubes using devices referred to as cheese knives. These are rectangular stainless steel frames that are either strung horizontally or vertically with stainless steel wires at 0.3 to 2 cm intervals. They may be used manually in open cheese vats or be designed as a mechanical knife/agitator in an enclosed, automated system. In either case, this is a crucial step in the process of cheese manufacture. The cutting of the curd into cubes greatly increases the surface area to facilitate whey expulsion and concurrent shrinkage of the curd particles.

5, 6, 7. Healing, Foreworking, Predrawing

There are a number of events that may occur between the time the fragile curd is first cut and the time cooking is initiated. First the curd is allowed to remain undisturbed for a short period of time. Whey, which escapes from the curd mass rapidly during cutting, becomes the dominant phase in the vat and the curd particles very slowly drop or settle toward the vat bottom. The firming-up of the curds or the forming of a semi-permeable membrane is frequently referred to as healing, although the term is probably scientifically incorrect. During this period of time the cheesemakers may wipe or "squeegee" the adhering curd from the sides of the vat to lessen "cook-on" during later events. Removal of curd from the vat sides prevents activation of clostridial spores that may be present within the adhering curd particles. Such activation may cause "stink spots" in high cook-out varieties like Swiss. After 10 to 15 min the curds are gently foreworked by manual agitation with a stainless steel paddle or shovel to prevent matting or balling of the curds. At the appropriate time some cheesemakers withdraw about 1/3 of the whey from the vat before cooking. This has at least three beneficial effects. Firstly, the whey at this stage is very sweet,

with perhaps 0.11% titrable acidity which is desirable in whey processing. Secondly, less energy is required in cooking with 1/3 of the vat contents having been removed. Thirdly, it speeds removal of the whey after cooking because there is 1/3 less volume to remove. Sometimes the predrawing is accomplished immediately after cooking.

8, 9. Cooking and Stirring-out

As earlier, the basic aim in cheesemaking is to remove a large portion of the water from milk. Prior to cooking, milk was transformed into a rigid coagulum and cut into thousands of small pieces, greatly increasing the surface area. The main objective of the cooking process is to further reduce the moisture content and firm up the curd. During cooking three main independent factors act on the curd to achieve this end. These are the effects of temperature pH and agitation.

Although the cooking process is extremely important in the manufacture of most cheese varieties, considerable variations in its execution are common from plant to plant producing the same variety or from variety to variety. The most significant variables are:

- (a) Elapsed time between cutting and cooking. This may vary from 5 to 45 min. With shorter time interval curd is softer, more delicate, and more susceptible to shatter. The longer the time interval, the greater will be the whey expulsion and curd shrinkage before cooking.
- (b) Rate of cook. A slow initial rate of temperature rise, such as 0.2 to 0.5°C for every 5 min, followed by a more rapid heating period (1.5 to 2°C/min) is used for manufacturing many cheeses. Such gradual elevation of temperature prevents "case-hardening" or the formation of a dense surface layer on the curd particles which inhibits whey expulsion.
- (c) Maximum cook temperature. During the cooking step the starter organisms multiply rapidly and metabolize lactose to lactic acid. Lactic acid promotes curd shrinkage and whey expulsion. In the manufacture of most cheese milk is inoculated below the optimum growth temperature of the starter flora. Therefore, as the cook temperature increases the metabolic activity of the starter organisms increases.

This is true up to a certain temperature which, if exceeded, decreases acid development. The specific temperature at which starter bacteria are inhibited depends on the starter species, i.e. lactic streptococci, lactobacilli, enterococci, or *Streptococcus thermophilus*. Blue cheese may be cooked to only 33°C, Cheddar to 39°C, Mozzarella to 48°C, and large curd Cottage cheese to as high as 55°C. If acid production is too fast, the rate of cook may be increased to slow down the starter activity. Care must be taken to prevent case hardening which encourages whey retention leading to an acidic, sour curd with a soft, high moisture core. Too high a cook temperature frequently produces a short, corky body in the finished cheese.

- (d) Rate of agitation. The rate of syneresis of the curd is markedly increased by agitation. Inadequate agitation during cooking results in curd particles lumping together, thus decreasing the effective surface area. These lumps resist expulsion of whey and tend to create high moisture areas in the resultant cheese. Conversely, rapid agitation tends to encourage whey expulsion.
- (e) Maintenance of cook temperature. Once the maximum temperature is attained the steam is shut off and the curds are stirred out for several minutes. Unless careful attention is paid to the vat temperature, it may drop 1-3°C. This decrease may delay attainment of the desired moisture level and acidity.

10, 11. Washing, Draining

In the early days of cheesemaking, curd was dipped from kettles or vats using cheesecloths. This gave rise to the term "dipping". Although that term is still in use, most manufacturers remove the whey leaving the curds in the vat. In some cases both curds and whey are pumped to one of a variety of devices which are used to separate the two components. These devices usually involve drain drums, drain belts, or drain vats which are designed to speed the separation process. If draining is achieved in the horizontal cheese vat, the curd is pushed back from the vat outlet (end gate) and a gate strainer is inserted to facilitate whey removal through the outlet without loss of curds.

12, 13. Matting, cheddaring

The make procedures for some cheeses do not include either matting or cheddaring while the manufacture of other cheeses includes only the matting step.

14. Milling

When the desired changes have been brought about by the cheddaring processes, the curd for Cheddar is subjected to milling. Milling also contributes to additional cooling of the curd before hooping to reduce the loss of fat in subsequent steps and decrease the incidence of seaminess in the finished curd.

15. Mixing-molding

In the case of Mozzarella, Scamorze, Provolone, and Pizza cheese, the milled curd is dropped into hot water (57-83°C) in a mixing-molding machine. The curd is stretched and mixed until uniform. The molten mass is then moved by an auger to the ports which discharge into the appropriate molds or hoops. The hoops are then placed in cold water to initiate the cooling and firming of the curd before brining. Molten curd may also be conveyed to forming machines which cut off a predetermined weight of hot curd which is then placed into small plastic or stainless steel molds to form the appropriate shape. Alternatively hot stretchable curd may be hand-worked into the appropriate shapes for certain markets.

16. Hooping

The placing of finished curds in hoops, forms, or molds is called hooping. These hoops are usually made of stainless steel or some type of plastic. They may accommodate from as little as 113.4 g to over 907.2 kg of curd. The hoops are also available in a wide variety of shapes such as: the popular 18.14kg rectangular block hoop used for Cheddar, Edam, and Colby; 2.27 to 3.18 kg short circular or square perforated hoops used for Blue; small bullet shaped unperforated forms used for Scamorze; the pipe-shaped forms used for Mozzarella and Provolone; cone-shaped pressing forms used for Sapsago; ball-shaped hoops with a raised central band used for Sardo; pear-shaped forms used for varieties of Provolone; and larger scale square or rectangular hooping and pressing vats that are used for such cheeses as Cheddar and Swiss.

Hooping is frequently regarded as a step where the cheese curd is transformed into a block loaf or slab which can be more easily handled.

17. Pressing

Once the cheese curds have reached the appropriate pH and moisture ranges many varieties require some sort of compression to fuse the particles together in a compact shape. This step is called pressing even when the compression is simply the result of the force of gravity.

18. Salting

Sodium chloride is incorporated into cheese by seven different ways or by combinations of any two or more of these individual procedures.

- (a) Dry salt may be added to the initial fluid milk such as in the manufacture of Domiati, an Egyptian pickled cheese.
- (b) Dry salt may be dusted on the curd in the vat just before hooping as is done with Cheddar.
- (c) Brine salt may be sprayed on the surface of curds in the vat before hooping. This is sometimes done with Cheddar.
- (d) Dry salt may be dusted or rubbed on the surface of the freshly formed cheese as in the case of Brie and Camembert.
- (e) Cheeses may be removed from the hoops and placed in 90-100% saturated salt brine tanks, i.e. Mozzarella, Swiss, and Romano.
- (f) Salt may be added to a creaming mixture or dressing which is then mixed with the cheese curd. Cottage cheese is an example.
- (g) Dry salt may be added, perhaps in conjunction with stabilizing gums, to fluid or semi-fluid cheese such as cream cheese just before final blending and packaging. This differs from item b), listed earlier, in that the salt is totally recovered in the finished cheese and it has no specific function in the actual manufacturing process other than flavor.

Salting of cheese achieves a variety of purposes depending on the type of cheese involved. In some cheeses it is primarily a

contributor to flavor, but in others it plays an intimate role in the make procedure. Salting of milled Cheddar curds promotes whey expulsion and moisture control. Increasing the time between salting and hooping results in lower moisture cheese. Floating of fresh Provolone in a cold brine containing liquid smoke facilitates the firming of the cheese body as it cools while simultaneously conveying a smoked flavor to the cheese. Brining also extends its shelf life by adding salt to and removing water from its surface regions. The curing room flora of Blue-veined cheeses is preferentially selected by the total accumulation of 3 to 5% salt in the loaves. Cheeses such as Feta and Domiati require quite high levels of salt very early in the manufacturing process. These cheeses are then pickled in a brine or whey-brine solution until ready for consumption. The preserving influence of cheese salt against certain micro flora is more readily understood if one were to realize that a cheese containing 2% salt and 37.5% moisture actually possesses an effective 5.33% salt in the moisture phase.

19. Ripening

This period of time is also referred to as aging, maturing, or curing. A complex array of biological, chemical, and physical reactions transforms the fresh curd into a finished cheese of desirable flavor and body. The starter flora and the enzymes thereof play a dominant role in the ripening of the cheese. Additional contributions are made by the continuing action of rennet and the enzymes of milk and adventitious microbes. Proteins are degraded, softening the body of the cheese. Simultaneously, peptides and amino acids are released, adding to the flavor profile. Fats are acted on by lipases releasing free fatty acids. Microbial metabolism generates aldehydes, alcohols, esters, and ketones which characterize particular cheese varieties. The microbial flora progressively changes as the available substrates become limiting and end products accumulate. The surface to volume ratio becomes important in the ripening of some cheeses because the interior portion becomes anaerobic quickly leaving only the surface for aerobes to express their presence. Temperature, curd moisture, humidity, microflora, and the length of the ripening time have a profound influence on the curing process.

The major manufacturing events and some typical corresponding analytical data for some of the most popular cheese

varieties are presented in the following Tables 5.14 through 5.21. The tables presented here are :

not to be considered as outlining the only approach resulting in good cheese. The events and their values often vary markedly from vat to vat within the same manufacturing plant. Greater variations generally occur among separate plants and among manufacturing countries.

Table 5.14 : Changes Occurring During the Manufacture of Bel Paese Cheese

<i>Step in the Manufacture of Cheese</i>	<i>Approximate Duration</i>	<i>Condition</i>			
		<i>Temperature (°C)</i>		<i>pH/Acidity</i>	
		<i>S</i>	<i>E</i>	<i>S</i>	<i>E</i>
1. Milk pasteurization	16 sec		72	6.6/0.15	6.6/0.15
2. Ripening after addition of about 1% starter	5 min	42	42	6.6/0.15	6.6/0.15
3. "Setting" coagulation by rennet 113 mL/453.6 kg	15-20 min	42	42	6.6/0.15	6.5/0.16
4. Cutting and healing of the curd (0.95 to 1.27cm ³)	10 min	42	42	6.6/0.16	6.4/0.10
5. Cooking	None				
6. Predrawing (optional)	5 min	42	42	6.4/0.10	6.4/0.10
7. "Stirring out"	5-10 min	42	42	6.4/0.10	6.3/0.11
8. "Dipping" or draining of the whey	5min	42	42	6.3/0.11	6.3/0.11
9. Hooping or filling molds	15min	42	40	6.3/0.11	6.3/0.11
10. Turning hoops every 10 to 20 min	3 to 4h	40	32	6.3/0.11	5.2-5.4
11. Brining	15 min to 4h depends on size of cheese	32	19	5.3	5.1
12. Curing	2 to 6 months	19	6		5.0

Table 5.15 : Changes occurring during the Manufacture of Blue Cheese

Step in the Manufacture of Cheese	Approximate Duration	Moisture Content (%)	Condition Temperature (°C)		pH/ acidity	
			S	E	S	E
1. Milk heat treatment	16 sec	8.75		72	6.6/0.155	
2. Ripening after 1 to 3% starter addition	30-45min	—	31	31	6.6/0.155	6.6/0.165
3. Setting-coagulation by rennet (85 mL/ 453.6 kg)	50-60min	-	31	31	6.6/0.155	6.6/0.18
4. Cutting (0.635 to 0.95 cm ³)	3min	-			6.5/0.18	6.5/0.12
5. Healing of the curd	5to 10 min	79.7			6.5/0.12	6.5/0.12
6. Foreworking	25-30 min	79.0			0.12/6.5	/6.45
7. Predrawing of part of the whey (optional)	5-10 min	76.0	31	31	0.14/6.48	0.14/6.48
8. Cooking	15-22 min	75.0	31	34	6.48	6.39
9. Stirring out	50 min	70.0	34	34	6.35	6.25
10. Predrawing of 1/3 of whey (optional)	5 min	68.0	34	34	6.25	6.25
Continued stirring out	25-30 min	67.0	34	34	6.25	6.1
11. Dipping or pumping curd from vat into hoops	8-15 min	65.0	34	33	6.1/0.15	6.0/0.155
12. Turning and dry salting of cheese in hoop	2h	62.0				0.38
	4h	56.0				5.05/0.63
	6h	51.0				
	24h	49.0				
13. Brine salting		50.0				
14. Curing						

Table 5.16 : Changes Occurring During the Manufacture of Cheddar Cheese

Step in the Manufacture of Cheese	Approximate Duration	Condition			
		Temperature (°C)		pH/Acidity	
		S	E	S	E
1. Milk heat treatment	16-20 sec varies to flash			71.6-73	6.6/0.165
2. Inoculation and ripening (0.5 to 1.25% starter)	45-60 min	31	31	6.6/0.165	6.50/0.175
3. Setting-coagulation by rennet (85-100 mL/453.6 kg)	30 min	31	31	6.50/0.175	6.45/0.18
4. Cutting and healing of the curd (0.64-0.95 cm ³)	10-30 min	31	31	6.45/0.18	6.4/0.115
5. Cooking	30-45 min	31	40-41	6.4/0.115	6.2/0.14
6. Whey removal or "dipping"	25 min	40-41	39-40	6.2/0.145	6.1/0.17
7. Matting or packaging of the curd	15 min	39-40	38-39	6.15/0.17	6.1/0.19
8. Cutting into slabs	5 min	38-39	37-38	6.1/0.19	6.1/0.19
9. "Cheddaring" or turning and piling of the slabs	100-120min	37-38	35-38	6.1/0.19	5.2/0.57
10. "Milling" - cutting into small strips (5.08×2.54×1.6 cm)	10 min	35-38	34-37	5.2/0.57	5.15/ 0.58
11. Salting-addition of about 21/4-3% NaCl to the milled curd in 3 applications	20-30 min	34-36	32	5.15/0.58	5.1/0.60
12. "Hooping" transferring salted curd to stainless steel molds	15-20 min	32	31-32	5.1	5.1
13. Pressing (includes prepress) (1.4 to 1.8 kg/ cm ²)	4-20h	30-32		5.1/	5.0
14. Packaging or boxing					
15. Curing or aging	2-12 months	2-15			5.0-5.2

Table 5.17 : Changes Occurring During the Manufacture of Short-Set Small Curd Cottage Cheese

Step in the Manufacture of Cheese	Approximate Duration	Condition		pH/Acidity	
		Temperature (°C)		S E	
1. Pasteurization of milk	30min or 16-25 sec	63	72-75	6.60	6.65
				0.16	
2. Ripening after 4-6% inoculation	60 min	31	31	6.10	5.95
				0.20	0.24
3. Setting-coagulation by acid and rennet (16-32 mL/453.6kg)	31/2-4 h	31	31	5.95	4.7
				0.24	0.54
4. Cutting and healing of the curd (0.3175-1.27 cm ³)	15-20 min	31	31	4.7/0.54	4.68/0.56
5. Cooking and stirring out	(1) 30 min	31	35	4.68	4.65
	(2) 30 min	—	39	0.56	0.58
	(3) 30 min	—	49-56		
6. Draining of the whey	30-40 min	50-52	49-51	4.65	4.65
7. Three step washing of the curd	variable (60-90 min)	49-56 32 15	32 15 3		4.5-4.6
8. Trenching, draining	variable (maybe 45-60 min)	3	3	4.5-4.6	4.5-4.6
9. Creaming and packaging	—	3	3	4.5-4.6	4.9-5.1

In the tables, the designation "S" denotes the "starting value" and "E" denotes "at the end of the process". Acidity values are expressed as % lactic acid. Values for the early stages of manufacture refer to milk or to whey, whereas those for the later stages refer to curd.

Table 5.18 : Changes Occurring During the Manufacture of Mozzarella Cheese

Step in the Manufacture of Cheese	Approximate Duration	Condition			
		Temperature (°C)		pH/Acidity	
		S	E	S	E
1. Milk heat treatment	16-18 sec	4	72		6.6
2. Inoculation and ripening	30-45min	31-35	31-35	6.6	6.45
3. Setting	30 min	31-35	31-35	6.45	6.35
4. Cutting of the coagulum and healing of the curd	10 min	31-35	31-35	6.35	6.32
5. Cooking	30 min	31-35	40-48	6.32	6.15
6. Stirring out	30 min	40-48	48-48	6.15	5.8
7. Predrawing	15 min	40-48	40-48	5.8	5.65
8. Continued stirring out	10-30 min	40-48	40-48	5.65	5.4
9A. Draining of the whey (rotary screen) or	40 min	40-48	40-48	5.4	5.3
9B. Alternative procedure of matting, cheddaring, and turning	40-46 min	40-48	40-48	5.4	5.3
10. Mixing-molding	45 min	40-48	57-68	5.3	5.25
11. Brining	1-14 h	57-68	variable	5.25	5.25
12. Packaging and storing	8-12 days	4-6	4-6	5.25	5.25

Table 5.19 : Changes Occurring During the Manufacture of Parmesan Cheese

Step in the Manufacture of Cheese	Approximate Duration	Condition			
		Temperature (°C)		pH/Acidity	
		S	E	S	E
1. Milk heat treatment	17sec	2-6	72		0.155/6.5
2. Ripening of milk after about 1% starter addition	10 to 30 min	33	33	0.155	0.18
3. Setting-coagulation by rennet (80 mL/ 453.6kg)	20 min	33	33	0.18	0.18
4. Cutting and healing of the curd	3 min	33	33	0.18	0.11
5. Foreworking	10 min	33	33	0.11	0.115

contd...

Table 5.19 – *contd...*

Step in the Manufacture of Cheese	Approximate Duration	Condition			
		Temperature (°C)		pH/Acidity	
		S	E	S	E
6. Cooking					
1st stage	34 min	33	42		0.12
2nd stage	26 min	42	52		0.14
7. Stirring out	5 to 10 min	52	52		
8. Hooping	5 to 10 min	52	49	0.17	
9. Pressing	45-180 min	49	42		
10. Brining at 10°C	15 days	42	10		
11. Curing					

Table 5.20 : Changes Occurring During the Manufacture of Romano Cheese

Step in the Manufacture of Cheese	Approximate Duration	Condition			
		Temperature (°C)		pH/Acidity	
		S	E	S	E
1. Milk heat treatment	16-25 sec	2-6	72		0.155
2. Ripening after addition of 1.0-1.5% starter	30-65 min	32	32	0.155	0.175
3. Setting-coagulation by rennet (85 mL/453.6 kg) simultaneous addition of kid lipase	20 min	32	32	0.175	—
4. Cutting and healing of the curd	5 min	32	32	0.115	0.115
5. Foreworking	10 min	32	32	0.115	0.12
6. Cooking	30 min or	32	48	0.12	0.14
1st stage	40 min	32	42	0.12	0.13
2nd stage	20 min	42	48	0.13	0.14
7. Stirring out	8 min	48	47	0.14	0.15
8. Dipping and hooping of the curd	10-15 min	47	45	0.15	0.155
9. Pressing of the curd includes turning and dressing	12-24 h	45	42		
10. Salting	20 days	24	10		
11. Curing	16-24 months	10	10		

Table 5.21 : Changes Occurring During the Manufacture of Rindless Block Swiss Cheese

<i>Step in the Manufacture of Cheese</i>	<i>Approximate Duration</i>	<i>Conditions</i>			
		<i>Temperature (°C)</i>		<i>pH/Acidity</i>	
		<i>S</i>	<i>E</i>	<i>S</i>	<i>E</i>
1. Milk heat treatment	15-18 sec	3-6	67.2-72.2	6.6/0.14	6.6/0.14
2. Ripening of milk	0.30 min	31.1-35	31.1-35	6.6/ 0.14	6.6/0.14
3. Rennetting (setting)	25-30 min	31.1-3.5	31.1-35	6.5/0.14	6.5/0.14
4. Cutting	15-20 min	31.1-3.5	31.1-35	6.5/0.14	6.5/0.085
5. Foreworking	30-60 min	31.1-35	31.1-35	6.5/0.085	6.5/0.088
6. Cooking	30-40 min	31.1-35	48.8-52.8	6.5/0.088	6.4/0.092
7. Stirring out	30-70 min	48.8-52.8	48-50.1	6.4/0.092	6.35/0.096
8. Dipping	10-15 min	48-50.1	48-50.1	6.35/0.12	6.3/0.12
9. Pressing	6-18 h	48-50.1	35-36.7	6.3/0.12	5.15-5.4
10. Brine salting	1-2 days		7.2-15	5.15-5.4	5.2-5.4
11. Drying	20 min (maybe up to 1 day)	7.2-15	7.2-15		
12. Wrapping					
13. Precooler	0-10 days	7.2-15	7.2-14	5.2-5.4	5.2-5.5
14. Warm room	2-7 weeks	7.2-14	21-25.5	5.2-5.5	5.5-5.7
15. Finished cooler	until sold	21-25.5	3-10	5.5-5.7	5.5-5.7

VI. Current Trends and Research Needs in Cheese Biotechnology

Although predictions and forecasts about the future of the cheese industry are difficult to make, judicious evaluation of current basic needs, economic pressures, and scientific developments allow some projections. The areas that need attention are:

1. Developments in the farm sector
2. Developments in engineering and mechanical devices.

6

Lactic Acid Fermentation of Cabbage and Cucumbers

From a historical point of view the fermentations of cabbage (*Brassica oleracea*) and cucumber (*Cucumis sativus*) has their inceptions in the Far East, notably China and India. As civilizations began to develop and expand into new hemispheres, the art of using lactic acid fermentation became firmly entrenched as an ideal method for preserving fruit and vegetable products.

The use of acidic fermentations by both the home and commercial food producer has been perpetuated because properly fermented products possess distinct and unique flavor characteristics. They are incapable of supporting the growth of microorganisms of public health significance and furthermore, the method permits commodities to be stored for prolonged periods of time without seriously impairing the physical and nutritional qualities of the product.

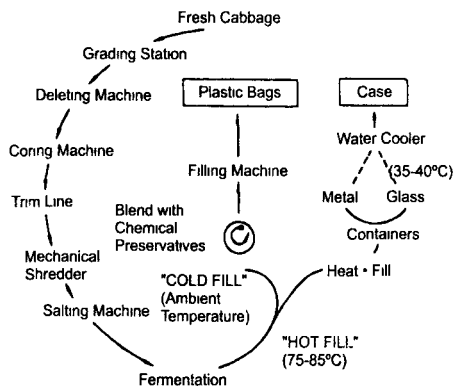


Fig. 6.1 : Flow sheet for the commercial production of sauerkraut.

The spectrum of horticultural commodities that undergoes acidic fermentations is quite extensive (green beans, beets, brussels sprouts, cabbage, carrots, cauliflower, celery, cucumber, olives, onions, peppers, green tomatoes, turnips etc.); however, only a few of these commodities are consumed in quantities of sufficient magnitude, to warrant their production on an extensive industrial scale. For example, in the United States only three commodities (cabbage, cucumbers and olives) provide significant contributions to the overall production volume of the fermented food industry.

Cabbage

The transformation of shredded cabbage to sauerkraut is, from a mechanical point of view, a very simple operation; however, from a biochemical and microbiological viewpoint the fermentation is enveloped in an array of complexities.

A schematic diagram depicting the steps involved in producing commercial sauerkraut is shown in Fig. 1

Cabbage Varietals

Crop distribution

Cabbage used for sauerkraut production is generally considered to be a "cold crop", i.e., its hardiness, proper development and maturity occur under those climatic conditions found within the geographical latitudes of, or equivalent.

New hybrids

Although the per capita consumption of sauerkraut, 0.64-0.72 kg, has remained quite constant throughout the past decade, the varieties of cabbage used to produce the final product have undergone significant changes. Many of those cultivars used for fermentations 10 to 15 years ago have been replaced by more vigorous species that are more amenable to current harvesting and ecological practices. For example, the need to tolerate the physical abuse incurred during mechanical harvesting and the environmental demands to reduce the generation of excessive volumes of spent fermentation brines has been resolved in part, by developing new hybrids that are compatible with the requisites of conservation. This is especially evident with the development of the "hi solids" species, i.e., cultivars genetically derived to contain lesser

amounts of native water. These varieties contain at least 20% more dry weight than their predecessors and provide similar increments in the yield of marketable products. Decreasing the moisture content of those varieties currently used in commerce has no adverse effects upon product quality; however, it markedly reduces the discharge volumes of waste effluent.

Mechanical Operations

1. Mechanical harvester

The modern mechanical harvester, capable of harvesting 7 to 14 tons of cabbage per hour, has essentially replaced the laborious process of cutting cabbage by hand. In Mexico more than 97% of the cabbage used for sauerkraut production is harvested by machine. Mechanical harvesting provides the processor with a continuous and ample supply of fresh cabbage and has thereby eliminated the need to stockpile cabbage for anticipated usage.

2. Grading

Once on the processor's premises the cabbage is subjected to rather intensive inspections. The criteria used for grading purposes include : type and extent of pest and insect damage; head size (minimum diameter : 14 cm); shape and firmness of head; length of internal core; presence of internal "spotting" and rot; and the amount of unusable leaf material (waste material).

3. Core removal

Following grading critique, the bulky outer leaves ("wrapper leaves") are removed in part by the mechanical deleafing machine consisting of a series of horizontal counter-rotatory rollers.

Following partial defoliation the heads are conveyed to the coring station. The coring machine is equipped with automatic vertical augers "drills" and removes the dense core matter. The removal of the tough core material is essential for producing a uniformly shredded cut.

4. Trim

Upon removal of the core, the heads proceed to the final trim line. At this point the skilled operators cut and remove extraneous green leaves, a step required for producing a sauerkraut that is

devoid of dark brown spots arising from chlorophyll blemish. In order to achieve this latter goal, 26 to 42% of the gross weight of fresh cabbage delivered to the processing plant is ultimately discarded as waste material.

5. Shredding

The shredded cabbage is prepared by passing the trimmed head through a mechanical slicer equipped with rotating knife edges affixed in a horizontal plane. A single shredding machine fitted with a circular blade 80 to 90 cm in diameter is capable of producing 80 to 90 tons of sliced cabbage per hour. The length and thickness of the shreds are determined by the distal setting of the blades and can be adjusted to those specifications established by the individual processor, usually about 0.10 cm in thickness.

6. Salting

The next step, the application of crystalline salt (food grade) to the shredded cabbage, is an extremely important factor because it directs the course of the fermentation and influences the quality of the final product. Salt serves as an important determinant because it :

- rapidly extracts from the plant cells those nutrients required to support microbial growth,
- inhibits the growth of some undesirable microorganisms,
- contributes to maintaining optimum textural properties,
- serves as a flavor ingredient in the final product.

The methods used for salting cabbage have, however, been more modernized. The fluming of cabbage in a brine solution has been totally abandoned and the "salting by hand" technique has been replaced for the most part, by automatic salting devices. These automatic salters are designed to dispense granular salt atop a thin layer of shredded cabbage. In principle the changing mass of cabbage passing a fixed point in the conveyor line is continuously measured and monitored by electronic sensors that subsequently proportion the salt on a differential weight basis. Under commercial processing conditions, salt is applied at the rate of 0.9 to 1.1 kg per 45.5 kg of shredded cabbage.

7. Conveyance

The use of push carts as a means of conveying salted cabbage to the fermentation tanks has been superseded for the most part by less labor-intensive method, such as endless belt conveyors and more recently, the pneumatic systems. The latter method may function in dual capacities that is, the salted cabbage is driven to the tanks through flexible conduits by positive air pressure and following fermentation, the product is recovered by using the negative pressure mode. Although the vortex motion created by the positive feed system serves to enhance the mixing actions of salt and shreds, the shear forces generated by these cyclonic motions frequently reduce the length of the strands to a "chopped" cut, a less desirable trait.

8 Fermentation tanks

Whereas wooden tanks, 20 to 70 capacities, have been traditionally used as fermentation vats, newly constructed units are today fabricated of reinforced concrete and provide storage volumes in excess of 180 tons of products.

The advent of synthetic coatings and the improved techniques for bonding these synthetic materials for bonding these synthetic materials to the interior surface of the concrete and wooden tanks provide liners that are impervious to attack by acidic brines. The use of inert liners not only facilitates sanitation but eliminates the need for keeping the wooden tanks filled with water, a step mandated to maintain the integrity of the tank following product removal.

Tank closure

Flexible plastic sheets placed atop the shredded cabbage serve as impermeable membranes for retaining the required liquid in the headspace. The fluid seal (water or brine, depth : 20 to 35 cm) readily assumes the geometrical configuration of the tank and thereby provides a tight closure for the maintenance of anaerobic conditions. In addition to serving as an effective cover, the seal provides the necessary weight to keep the shreds immersed in the brine. An inadequate brine overlay invariably produces irreversible discoloration and serious losses in textural integrity at the brine-shred interface.

Fermentation

Since the inherent microbial population of fresh cabbage is extremely diverse, both chemical and physical factors must be utilized to retard or limit the growth activities of undesirable genera while concomitantly favoring the growth responses of the desirable species, namely the lactic acid bacteria. These requirements are achieved in part by anaerobiosis, the judicious use of salt, and temperature effects.

From a practical point, anaerobiosis is self-achieved via the respiratory action of the shredded cabbage and the indigenous microbial population. This imposed restraint limits the metabolic activities of aerobic genera, notably *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, molds, and oxidative yeast so frequently associated with vegetable products. Needless to say, anaerobiosis is only one of several factors that negates the growth responses of some undesirable species.

Other factors that warrant consideration include pH and acid production; however, the exact contributions of these functions as independent effectors of growth are difficult to assess because they function in concert. For example, the populations of some microbial species may wane in response to increased hydrogen-ion concentrations (decrease in pH) or ionic species (acids), whereas other microorganisms are less vulnerable to, or prefer more acidic conditions (lactic acid bacteria). Such shifts in populations are especially apparent throughout the course of the sauerkraut fermentations.

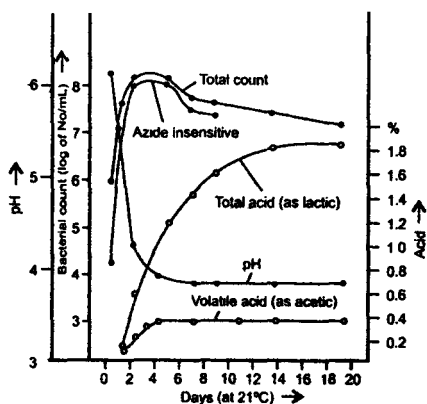


Fig. 6.2 : Fermentation pattern of sauerkraut.

During the early phase of the fermentation (Fig. 16.2), 99% of the total viable count (10^6 Cells/mL) was comprised of azide-sensitive bacteria. [Azide is a potent inhibitor of the cytochrome system and restricts the growth of aerobic (non-lactic acid) bacteria.] Following two days incubation, however, the trends in population were completely reversed in that, within this total population (10^8 cells/mL), more than 90% of the viable cells were lactic acid bacteria.

Further evidence that the lactic acid bacteria were the dominant group is reflected in the marked reductions of the pH, that is from 6.2 to 4.2 within 48 hours, then a subsequent reduction to pH 3.7 following two days of incubation. Although the rapid decrease in pH, i.e., 6.2 to 4.2 during the initial phase of fermentation undoubtedly served to limit the growth of acid-intolerant species, it likewise reflects the weak buffering capacities of cabbage.

The changes in the composition acids during the course of the fermentation (Fig. 6.2) are attributable to the differences in the metabolic pathways used for the dissimilation of carbohydrates by the heterolactic and homolactic acid bacteria.

The lactic acid bacteria chiefly responsible for heterolactic fermentations are *Leuconostoc mesenteroides* and *Lactobacillus brevis*; of these two species, *Leuconostoc mesenteroides* is the most prominent and active during the initial aspects of the fermentation. The reason that *L. mesenteroides* is able to play such a dominant role in the early phase of fermentations does not reside necessarily in its numerical superiority but rather, that it displays the shortest comparative lag and generation times of the lactic acid bacteria associated with the sauerkraut fermentation.

It may also be observed (Fig. 16.2) that the production of volatile acidity (acetic acid) is significantly curtailed following 4 days fermentation. The reduction in volatile acid production occurs because *L. mesenteroides* is not able to tolerate the additional acidity and subsequent reductions in pH generated through the actions of the homofermentative species namely, *Lactobacillus plantarum* and *Pediococcus cerevisiae*.

The contributions provided by each of the participating species to the general flavor quality of sauerkraut are difficult to define. By using pure cultures it was shown that no singular species is capable of producing an acceptable sauerkraut.

Since the aroma and flavor profiles of sauerkraut are extremely complex and a consortium of cultures is required to achieve the desired results, starter cultures have not been deemed to be advantageous for commercial fermentations and consequently, the practice of adding selective cultures in order to modify or enhance fermentations has not met wide acceptance in the industry.

Flavor and color characteristics may also be altered by creating unbalanced homolactic or heterolactic populations. Such shifts in populations may occur in response to variations in temperatures and salt concentrations. For example, reduced salt concentrations, less than 2%, favor the growth of the heterofermentative *Leuconostocs*, whereas salt levels in excess of 3% retard the growth of these species. These responses to salt concentrations permit the more rapid emergence of the more salt tolerant species, such as the homolactic *pediococci* and *lactobacilli*. A dominance of homolactic species during the early stages of the fermentation can influence the flavor characteristics of the final product. The flavor imbalance is due in part to the presence of elevated levels of lactic acid which imparts a "sour" flavor and a diminution in bouquet resulting from an inadequate production of volatile neutral components.

Reduced temperatures, 10°C or less, are not conducive to the growth of most lactic acid bacteria, but *Leuconostoc mesenteroides* has the capability of growing at 7°C. Although the growth rate is markedly reduced, this species is capable of generating sufficient amounts of acidity to impede the growth of undesirable psychrophiles. However, at 18°C, the growth rates of *L. mesenteroides* are accelerated and the minimum temperature barriers imposed upon the *lactobacilli* no longer becomes a growth restriction. Although *L. mesenteroides* still plays an active role in initiating the fermentation, the increase in temperature likewise supports the rapid growth interactions among the numerous species of lactic acid bacteria. Thus, at 18°C, the rate of lactic acid production is increased nearly 8 fold and the required sequential growth patterns become established and provide the optimum balances in the hetero and homo-fermentations.

Deleterious effects such as losses in color (browning), ascorbic acid content, and degradation in textural and flavor characteristics are more frequently encountered when the temperatures of fermentation exceed 30°C. In part, this may be attributed to the

dominant and rapid growth responses of the homo-fermentative species to the elevated temperature and the resulting inability of the heterofermenters to serve effectively as catalysts in promoting the microbial reductions and transformations of critical chemical components. The adverse effect of higher temperatures upon product quality may be a reason why only a limited number of sauerkraut factories are located in the warmer Southern regions of India.

Product Defects

Whereas the physical and chemical interactions between salt and cabbage are at best, only poorly understood, it is quite apparent that the textural characteristics of the final product are influenced by salt concentration. For example, salt applied to cabbage at concentrations less than 1.8% (w/w) will invariably produce a sauerkraut of "soft" texture, whereas applications in excess of 2.5% begin to impart fibrous and tough textural properties to the product. Although the reasons for these extreme responses to salt concentrations are not readily apparent, it suggests that salt may serve to partially inactivate pectinolytic enzymes inherent in cabbage and/or reduce the pectinase activities of salt-intolerant organisms.

1. "Off" flavor

The formation of excessive amounts of short-chained fatty acids, especially butyric and propionic acids, are responsible for this defect.

2. Color defects

Visible and highly objectionable color defects may be produced by the growth of yeasts or bacteria. For example, "pink kraut" resulting from the growth of pigmented yeast, presumably *Rhodotorula* results from the failure of achieving uniform salt distribution. However, since the advent of automatic salting machines and the recognition of the importance of salt in fermentations, this defect is rarely encountered in commercial fermentations today.

Red color may also be produced by a non-pigmented bacterium, *Lactobacillus brevis*. Under conditions of controlled pH, *L. brevis* produced in cabbage juice a magenta color that

progressively deteriorates to a mottled-gray discoloration. Color formation is catalyzed by aeration and inhibited by naturally occurring reductants of cabbage, such as cysteine and ascorbic acid.

3. Processing defects

Although the above examples of defects are caused by microbial actions, it should be borne in mind that improper acid-salt ratios in the final product may affect flavor quality. In general a finished canned product containing 1.0 to 1.8% acid should contain sufficient amount of salt to provide an acid to salt ratio of 1:1.3. Excessive deviations from this acid-salt ratios, usually result in producing a product of reduced quality.

Processing

1. Bulk sauerkraut

Because of potential spoilage by fermenting yeasts, fresh or bulk (non-processed) sauerkraut is not packaged in sealed containers; therefore, this type of product is not readily available to the consumer in the United States. Only a very limited volume, less than 1% of the total production, is distributed as bulk sauerkraut and subsequently destined for retail sales.

To provide the required shelf life, sauerkraut is preserved by using two well established processes namely, the applications of heat or chemical additives.

2. "Hot fill" method

In many processing plants, the thermal screw heater has replaced the immersion-type pre-cooker. With the thermal screw heater, the fresh product is forced to migrate by auger action through an inclined tunnel permeated with steam. The rate of product feed and the residence time in the heater, approximately 3 min, are adjusted to provide a temperature of 77 to 82°C to the emerging product.

In the immersion-type system, the fermented sauerkraut is added to heated brine (74°C) contained in a large rectangular tank. The product, immersed in the hot brine, is agitated by revolving reels and simultaneously advanced to the exit conveyor by endless chain drives. Since it is more difficult to achieve a uniform mixing of the

shredded masses in the pre-cooker, the residence time is subject to variation and may produce an "overcooked" product. The latter process may produce objectionable alternations in flavor, color, and textural profiles of the final product.

In either case and following the heating step, the product is conveyed to a mechanical filling machine that is likewise maintained at temperatures of 74 to 79 °C and where the glass or metal containers are filled with hot product brine, hence the term "hot fill".

Following closure, the containers pass through a water cooler where upon existing the internal temperature of the product has been reduced to 38 to 43 °C. The containers are then placed in shipping cartons and are available for distribution.

Nearly 85% of the total production volume of sauerkraut is processed in glass and metal containers; of this volume, 90% is packaged in metal containers.

Sauerkraut packed in enamel-lined cans has an anticipated shelf life of 18 to 30 months. The shorter shelf life for the glasspack product, 12 months, is attributed to degradation in color resulting from exposure to light.

3. Chemical preservatives

The use of chemical additives such as sodium benzoate and potassium metabisulfite preclude the use of heat as a means of preserving sauerkraut.

In this "cold" method, a thick slurry of fresh sauerkraut and brine is drawn by vacuum into a mechanical filling machine specifically designed to handle the long shreds of sauerkraut.

Flexible plastic bags, formed on-line, are filled to about 75% volume capacity with the slurried product. The native brine containing sodium benzoate and potassium metabisulfite is then added to the product and, the bag is sealed by a thermal weld.

Legal restrictions limit the usage of benzoate to 0.1% (w/w), whereas the concentrations of SO₂ are of such limitations as to conform to the code established for Good Manufacturing Practices.

Sauerkraut packaged in plastic bags and kept under refrigeration (5 °C) has an anticipated shelf life of 8 to 12 months.

Cucumbers

A. Production and Consumption

Based upon the total production of vegetables used for processing, cucumbers, rank as the 4th leading truck crop in India. In 1981 the tonnage of cucumbers grown for pickle production exceeded 575000 tons.

B. Varietals and Harvesting

Newly derived varietals of cucumbers used in fermentations today have been genetically developed and specifically selected to provide; greater uniformities in the size, shape, color, and texture of the mature fruit; accelerated growth rates (decreased time required to reach maturation); and, last but not least, increase in harvest yields. In regards to harvesting, improvements in harvester design and the development of varietals of fruit that are compatible for use in the "once-over" harvesting method are advances that have contributed in part, to reducing the needs required by the labor intensive handpicking process. For example, as practiced in Michigan more than 90% of the acreage was harvested by machine in recent years. Although the once-over method may be feasible to use, some growers in order to increase the yield of marketable fruit have elected to pre-pick the crop prior to final harvest by machine. Undoubtedly the efficiency and use of mechanical harvesters will increase through improved design and development of multipick machines.

C. Grading

Once harvested, the fresh cucumbers are especially vulnerable to attack by the naturally occurring heterogenous microbial population indigenous to the fruit. To avoid postharvest deterioration the cucumbers are transported to the processing plants as soon as possible or alternatively cooled (refrigeration, hydrocoolers), and stored for short periods of time prior to transit.

At the plant the injured and deformed cucumbers are removed by sorting. Grading, i.e., size selection based on the diameter of the fruit, prior to brining is discretionary; however because of labor costs, field run (mixed) sizes are commonly used, and then resorted as brine-stock.

D. Fermentation

Pickles are packed under 2 general types of classification; the cured type, and the fresh pack. The former type is obtained by a completed lactic acid fermentation in a salt brine that may contain dill, herbs or other flavoring agents, whereas the latter pack is prepared from unfermented or slightly fermented cucumbers and subsequently packed in vinegar containing various flavoring ingredients. Each of these types, depending on the combinations of sugar, spices etc. that are used, may be further divided into numerous and descriptive subtypes, for example, dill, sour, sweet, etc.

Although pickles prepared by fermentation (cured-type) have served as the mainstay in the industry for many years, the fresh type pickle products have achieved such a high degree of consumer's acceptance that the two types currently share near-equal volumes of sales.

Since cucumbers used for fermentation are a seasonal crop and are intolerant of extended periods of storage, the fermentation serves as an ideal method for preserving this highly perishable product. Two approaches based upon differing salt concentration, i.e., salt stock or low salt methods serve to impart the required preservative actions.

1. Salt stock

A 40° salometer brine (40% saturated salt solution) is added to wooden tanks ranging in capacities from 100 to 1000 bushels (2½ to 25 tons). This brine, 30 to 90 cm in depth, serves to form a cushion, thereby reducing excessive crushing of the fruit upon filling the tank. When the tank is full, the cucumbers are covered with a "false" head constructed of slatted wooden boards that is keyed down to keep the cucumbers totally immersed in the brine. The headboards are constructed so as to provide numerous avenues for the fermentation gases to escape. Since the frequently unsheltered and open tanks are subject to the prevailing conditions of the natural environs [dilution (rainwater), evaporation of brines, insect and rodent infestation, etc.,] additional brine (40° salometer) may be added to provide a constant brine level that is 10 to 15 cm above the head-boards.

To achieve equalization of salt concentrations, the brines are periodically recirculated by pumping action. As the lactic acid bacterial population becomes established, the salt concentration is gradually increased to about 60° salometer. This elevated salt level allows the pickles to be stored safely for at least one year and likewise, prevents freezing of the stock in frigid areas.

(a) Development of flora

The microbial flora of cucumbers is composed, as in the case of cabbage, of a diverse population. However, the pattern of developmental growth and the participating species may differ from those described in the sauerkraut fermentation. These differences in response may be related to the usage of higher salt concentration, the chemical composition of cucumbers, and the use of fermentation tanks that are less than anaerobic. (Protective covers, similar to those used in covering vats of sauerkraut, have recently been introduced and are being gainfully used in the pickle industry. The expanded use of flexible covers or the fabrication of tanks similar in structure to those units used in the fermentation of olives, may significantly contribute to improving the quality and yield of product.)

Since the initial salt concentration frequently used in pickle fermentations is 2 to 3-fold greater than that used in the sauerkraut fermentation, the course of the fermentation becomes much more influenced by salt tolerant species. Three groups of microorganisms, i.e., coliform, lactic acid bacteria and yeasts are active during the course of this fermentation. The coliform bacteria consisting of two types, those tolerant of lower salt levels (5%), and the more halophilic species *Enterobacter*, formerly called *Aerobacter*, are capable of generating gases in brines containing 10 to 15% salt. If the growth of these species is not restricted by sufficient acid development, as generated by the lactic acid bacteria, undesirable and excessive gaseous fermentations (CO_2 and H_2) may occur.

The lactic acid bacteria chiefly responsible for acid production are *Lactobacillus plantarum*, *L. brevis*, and *Pediococcus cerevisiae*. Although *Leuconostoc mesenteroides* play a major role in the sauerkraut fermentation, this species is less contributory to the pickle fermentation. The inability of this latter species to compete more actively is due undoubtedly to the increased brine concentrations and the concomitant reduction in pH resulting from the competitive actions of homolactic acid bacteria.

(b) Defects

One defect that may manifest itself as a result of the fermentation is the formation of "bloater or floater" types pickles. In this malady the seeds and flesh within the central area of the pickle become dishevelled and displaced resulting in cavity formation. This void becomes filled with CO₂ thereby imparting buoyancy to the fruit, and hence the term "bloater". Although this defect produces an inferior pickle (relish grade), losses in product quality can be markedly minimized by using "controlled" fermentations.

(c) Controlled fermentation

Using this procedure the fresh fruit is thoroughly washed with water then covered with brine contain acetic acid and chlorine. The salinity is maintained at a pre-determined concentration; the quantity of salt used depends on the size of the cucumbers being brined.

Following 1 to 2 days incubation and prior to increasing the salt levels, sodium acetate is added to the brined stock. This buffered brine, subsequently inoculated with selective strains of *Lactobacillus plantarum* and *Pediococcus cerevisiae* ensures rapid carbohydrate utilization by these homofermentative bacteria. The resulting deprivation of substrate limits the growth activities of gas producing bacteria and fermenting yeasts, and likewise, reduces a major carbon source available for the potential generation of CO₂.

Carbohydrates are not the only substrates that serve as donors of CO₂. *L. plantarum*, the species used in the controlled fermentation process, converted the malic acid of cucumber to lactic acid and CO₂. Thus, the malolactic fermentation may also contribute to the evolution of CO₂.

2. Dill pickles

Genuine dill pickles are fermented in brines containing dill and spices. Since these pickles are fermented in cover brines, of relatively low salt concentrations, 3 to 5% the emergence of the lactic acid bacterial flora is responsive to the interactions of salt concentration and temperature and therefore, bears resemblance to that described for the sauerkraut fermentation. For example, at lower temperature, i.e., 18 °C or less, the fermentation pattern is initiated by the growth

of *Leuconostoc mesenteroides* and is terminated by the homofermentative species. However, as the temperature is increased the growth response of *L. mesenteroides* becomes less apparent and is displaced by a homolactic population.

The optimum temperature for the fermentation is 20 to 26 °C and the time required to produce a fully fermented and cured pickle of acceptable quality is about 8 weeks. The brine, pH 3.2 to 3.6, contains 0.7 to 1.2% acid, expressed as lactic acid.

Another type of low salt dill pickle, sometimes referred to as "Refrigerated", "Half Sour", or "Fresh Pack", is prepared in a manner similar to that used for genuine dill pickles except that the fermentation is carried out under controlled and reduced temperature (4-5°C).

The acidified brines used to cover the cucumbers are of such composition as to provide equilibrated salt concentrations of about 3% and pH values less than 4.6.

During storage the lactic acid concentration increases very slowly and, following 4 to 6 months incubation may reach acid levels of 0.4 to 0.6% expressed as lactic acid. The increase in acidity can undoubtedly be attributed to the growth responses of the lower temperature-tolerant.

3. Spoilage

In addition to being susceptible to bloating, salt stock and genuine dill types are especially prone to softening. The losses in texture may result from the pectinolytic enzymes from 3 distinct origins; bacterial, mycotic and those indigenous to cucumber.

Workers have extensively investigated the pectolytic properties of Gram negative and Gram positive bacteria causing softening of cucumbers. In brief, the genera *Achromobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, and *Erwinia* exert pectinolytic actions within the pH range 5.0-5.5 and in brines containing 5 to 8% salt. These species, therefore, are potential spoilage organisms during the early phase of the fermentation. On the other hand, molds and yeasts (oxidative and fermentative) are capable of growing throughout a broad spectrum of hydrogen-ion and saline concentrations and consequently are persistent sources of potential spoilage.

Some fermentative yeasts, such as *Rhizodotorula* and *Saccharomyces* in addition to generating CO₂ (a gas incriminated in

causing bloating), also produce polygalacturonase, an enzyme capable of degrading pectins. Strains of yeast are inhibited when the salt concentration exceeds 5%; this may offer an explanation why fermentative yeasts are more prevalent in brines containing lesser amounts of salt.

Yeasts representative of genera *Debaryomyces*, *Candida*, *Endomycopsis*, and *Zygosaccharomyces* grow luxuriantly on the surface of the brines. As a result of the utilization of lactic acid by these yeasts, the pH of the acidic brines increases thereby permitting the growth and establishment of an undesirable microflora.

Although softening of fruit tissues is attributable in part, to the actions of extracellular enzymes of microbial origin, endoenzymes native to cucumbers must also contribute to the autodegradation of textural structure. For example, studies on the characterization of cucumber endopolygalacturonase showed that the degrading action of this homogeneous enzyme located in seed cavity tissue increased more than 20-fold during maturation.

4. Preservation

Following fermentation the pickles (salt stock) are subjected to several changes of water to remove excessive amounts of salt. The "refreshed" pickles are placed in containers covered with brines containing condiments and spices that are appropriate for the type of pickle that is desired.

The finished product, derived from refreshed salt stock or genuine dill types is pasteurized at 78.3 °C for 15 minutes, then rapidly cooled.

In the case of the refrigerated type, the lightly fermented pickle is covered with an appropriate brine containing spices and sodium benzoate (the latter not to exceed 0.1% [w/w]) and stored at reduced temperature, 4 to 7°C.

2, 3-Butanediol

Biomass conversion gives both pentoses and hexoses as products. While the hexoses (primarily glucose) are readily fermented, routes for pentose fermentation are still being developed. Hence, pentoses represent a potentially significant source of sugars with xylose being the major product.

Properties

Stereochemical Configurations

There are three isomeric forms of 2,3-BD : D-(-), L-(+) and *meso* (Figure 1). Both the *meso* and (±) forms exist, to a considerable extent, in the conformation in which the hydroxy groups are *gauche* to each other (Figure 2). This conformation is favored because of the energy gained in the formation of the hydrogen bond. As a result, the methyl groups are *gauche* in the *meso* form and *anti* in the optically active form. Hence, the optically active form of the isomer is more stable than the *meso* form.

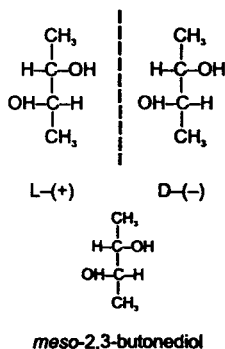


Fig. 7.1 : Three stereoisomers of 2,3-butanediol.

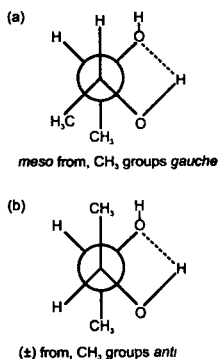


Fig. 7.2 : *Anti* and *gauche* forms of 2,3-butanediol

Physical Properties

The water/butanediol equilibrium data show no azeotrope. The distillation of 2,3-butanediol removed the water overhead with 2,3-butanediol being the bottom product. While the boiling points of *meso* (181–182 °C), D (179–180 °C) and racemic (177 °C) 2,3-BD are slightly different, they all have boiling points much higher than water. Hence, the recovery of 2,3-BD from fermentation broth requires a large quantity of water to be evaporated. In practice, it was found that an excess of lime must be added to filtered fermentation liquor if 90% recovery were to be obtained through steam distillation. If the liquor was not filtered and lime was added, recovery was only 50% due to decomposition. Process economics would dictate that essentially complete recovery be attained. An alternate approach suggested is liquid-liquid extraction, with *n*-butanol being a preferred extractant. Butanediol is then recovered as a bottom product in a subsequent distillation step with butanol being recycled.

A major challenge in the economic production of 2,3-butanediol would still appear to be in separation of 2,3-BD from water (or fermentation broth) in an efficient manner.

Fermentation

Substrates

The single major cost in most biomass conversion process appears to be the substrate cost. Hence, the availability of an inexpensive carbohydrate material is essential for developing an

economical fermentation process for production of 2,3-BD. Substrates suitable for the *K. oxytoca* fermentation include molasses, enzymatically hydrolyzed cereal mash, acid hydrolyzed starch and wheat, wood hydrolysates and sulfite waste liquor. *B. polymyxa* secretes amylolytic enzymes and hence is able to utilize cornstarch and whole grain mash of wheat. The high post-fermentation solids content of whole grains impairs the recovery of 2,3-BD from the fermentation broth, and hence renders the use of such mash to be less than optimum.

K. oxytoca and *B. polymyxa* are both able to utilize pentoses as well as hexoses. This is of considerable practical importance since hydrolysate from biomass materials can have pentose : glucose ratios of 1:1.5. As a result, almost all of the sugar present in hemicellulose and cellulose hydrolysates can be converted to 2,3-BD.

Fermentation Conditions

The optimum pH for butanediol production is in the range pH 5.0-6.0. The specific substrate utilization rate is maximum at pH 5.5. Above pH 6 the activity of one of the key enzymes in the butanediol pathway decreases sharply.

The optimum temperature for growth, sugar uptake and butanediol production is 37 °C. It is interesting to note that while 37 °C appear to be the best temperature for growth and fermentation, the highest butanediol concentration reported in the literature was achieved at 30 °C.

The most important variable affecting the butanediol yield and the fermentation rate is the availability of oxygen. Although 2,3-BD is a product of anaerobic metabolism, aeration has been shown to enhance its production. Workers suggested that aeration increases the butanediol productivity by increasing the cell concentration. However, too much aeration can decrease the yield of 2,3-BD. *K. oxytoca* is a facultative anaerobe which is able to obtain the energy it needs for growth by two different pathways : respiration and 'fermentation' (Figure 3). During oxygen limited growth (DOT < 5 mm Hg), both energy producing pathways are active simultaneously and the yield of butanediol depends on the relative activities of each of the three pathways depicted in Figure 7.3. The butanediol yield can be maximized by minimizing the oxygen availability because this limits respiration. However, with a small

oxygen supply, little cell mass is produced and, therefore, the conversion rates are slow. The butanediol production rate can be maximized by increasing the oxygen supply rate because this leads to a higher cell density.

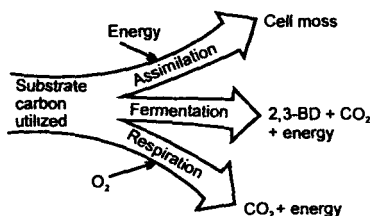


Fig. 7.3 : Pathways of substrate utilization for *K. oxytoca*.

The oxygen supply rate is also important because the ratio of oxygen demand to oxygen supply can control the proportions of various metabolites produced. Fermentation products excreted by *K. oxytoca*, other than 2,3-BD, include acetoin, ethanol, acetate and others. In the absence of oxygen, ethanol is produced in approximately equimolar amounts with 2,3-BD. The presence of some oxygen appears to inhibit ethanol production. Increasing the oxygen supply rate toward the value of the potential oxygen demand results in an increase in the acetoin : butanediol ratio. If the oxygen supply rate exceeds the microbial oxygen demand, oxygen is no longer limiting and the only products of sugar metabolism are cell mass and carbon dioxide. Therefore, in order to maximize butanediol production, a limited but non-zero supply of oxygen is required.

Substrate concentrations used for this fermentation are generally in the range of 5-10%. Substrates commonly used in industrial-scale fermentations are usually diluted to even lower sugar concentrations. In experiments employing D-xylose as the sole carbon source, metabolic rates decreased significantly at xylose concentrations greater than 20 g l⁻¹. Indeed, when the xylose concentration exceeded 160 g l⁻¹, no growth at all was observed. This apparent substrate inhibition may be explained by the dependence of the microbial growth rate on the water activity. When any solute decreases the water activity of the fermentation broth, the growth rate of *Klebsiella* also decreases. The low osmotic tolerance of *K. oxytoca* may be an important factor to consider in developing processes utilizing natural carbohydrate sources.

Fermentation Yields and Rates

The theoretical maximum yield of 2,3-butanediol from glucose is 0.50 g g^{-1} . The yield is the same from pentoses, which are converted to glyceraldehyde 3-phosphate by the pentose phosphate pathway. Actual butanediol yields obtained using *K. oxytoca* can exceed 80-90% of theory.

The efficiency of the 2,3-butanediol fermentation can be judged by the product yield from sugar, the final butanediol concentration and the volumetric butanediol production rate. Table 1 lists values for these performance indexes that have been reported for various types of reactor configurations. These results were obtained using glucose or sucrose as the carbon source. When substrates of industrial interest are used, the butanediol yield is usually slightly lower.

The fed-batch reactor has the capability of producing a high final butanediol concentration while minimizing the effects of initial substrate inhibition and final product inhibition. With continuous reactors; much higher rates are possible; however, product inhibition and incomplete substrate utilization are problem. The two-stage continuous culture system is outstanding in its ability to rapidly produce a high butanediol concentration with a good yield from sugar. Another promising system employs immobilized cells in an attempt to increase the conversion efficiency.

Table 7.1 : Comparison of Yields for Different Types of Fermentation

Reactor type	Butanediol yield (g g^{-1})	Butanediol concentration (g l^{-1})	Butanediol productivity ($\text{g l}^{-1}\text{h}^{-1}$)
Batch	0.43	65	1.6
Fed-batch	0.37	99	0.9
Continuous	0.32	30	3.0
Two-stage continuous	0.46	67	2.7
Immobilized cells	0.25	3.4	1.3

Biochemistry

The major intermediates in the conversion of a pentose or hexose to 2,3-butanediol are shown in Figure 7.4. The last step in the biological pathway of the fermentation involves the reduction

of acetoin (2-hydroxy-2-butanone) to 2,3-butanediol. While 2,3-BD has two assymetrical centers, acetoin has only one, and hence two stereoisomeric forms, D-(-) and L-(+). Hence, the reduction of acetoin to 2,3-BD may involve as many as to substrates and three products.

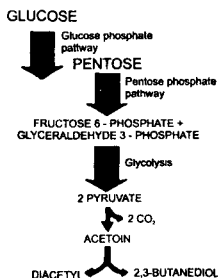


Fig. 7.4 : Major intermediates in conversion of a pentose or a hexose to 2,3-butanediol. Heavy arrows represent reactions of the pentose phosphate and glycolytic pathway. Light arrows indicate individual reactions.

Workers reported that *K. oxytoca* forms acetoin from pyruvate by the action of two enzymes. An acetolactate-forming enzyme catalyzes the condensation of two pyruvate molecules combined with a single decarboxylation to yield acetolactate and CO₂. The decarboxylase is specific for the dextrorotatory isomer and the product is the levorotatory isomer of acetoin (D-(-)-acetoin). Both the decarboxylase and acetolactate-forming enzymes have been partially purified and characterized.

The acetoin can be oxidized to 2,3-butanedione (diacetyl) by O₂ present in the fermentation medium, or enzymatically reduced (with NADH as a cofactor) to 2,3-BD. There stereoisomers are possible; indeed, for 40 years they have been known to exist in the fermentation broth. The isolmeric composition varies with the microorganism used in the fermentation.

Others reported that *K. oxytoca* produced a mixture of *meso* and L-(+)-2,3-BD. *Aeromonas hydrophilia* and *Aerobacillus polymyxa* produce only D-(-)-2,3-BD. Bacterial oxidation of 2,3-BD is a function of the stereo configuration as summarized in Table 2.

A mechanism for the formation of 2,3-BD stereoisomers by microorganisms was proposed. These investigators postulated the existence of two 2,3-BD dehydrogenases : (1) a dehydrogenase

catalyzing reduction of D(-)-acetoin to *meso*-2,3-BD, and (2) dehydrogenase catalyzing reduction of D(-)-acetoin to D(-)-2,3 BD. The existence of an acetoin racemase was mentioned as a possibility. These investigators did not consider the formation of L-(+)-2,3-BD. Experimental data were not provided for the model.

Scientists proposed a model for the formation of 2,3-BD stereoisomers from acetoin (Figure 7.5), based on the observed optical rotation of acetoin produced from pyruvate, the composition of 2,3-BD stereoisomers formed in the fermentation, and rates of oxidation of 2,3-BD stereoisomers. The model proposed the existence of three enzymes : an acetoin racemase, L-(+)-2,3-BD dehydrogenase and D-(-)-2,3-BD dehydrogenase. The dehydrogenases were said to be non-specific with respect to acetoin stereoisomers. That is,they would accept either acetoin isomer as substrate, but the reaction product would still be dependent on the acetoin isomer reduced. For example, the L-(+)-dehydrogenase would reduce L-(+)-acetoin to L-(+)-2,3-BD and D-(-)-acetoin to *meso*-2,3-BD.

Table 7.2 : Bacterial Oxidation of 2,3-Butanediol by Various Microorganisms.

Microorganism	Observation
<i>K. oxytoca</i>	<i>meso</i> and L-(+) are oxidized
<i>A. polymyxa</i>	D-(-) oxidized faster than <i>meso</i>
<i>A. hydrophila</i>	Only <i>meso</i> -2,3-BD is oxidized
<i>Pseudomonas fluorescens</i>	All three isomers of 2,3-BD oxidized

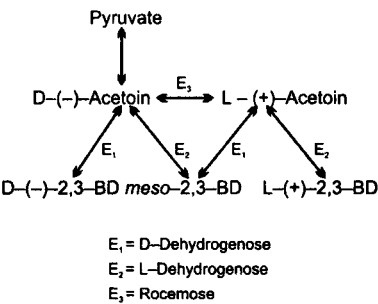
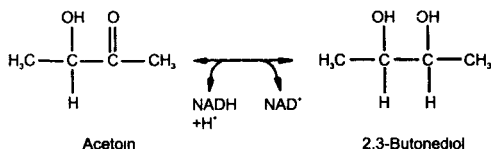


Table 7.5 : Mechanism for the formation of 2,3-butanediol stereoisomers by bacteria as proposed. For *K. oxytoca*, the presence of an acetoin racemase and L-(+)-2,3-butanediol dehydrogenase was proposed.

For *K. oxytoca* workers proposed the presence of acetoin racemase and the L-(+)-2,3-BD dehydrogenase. While they were unable to document the present of acetoin racemase in acetone-dried preparations of *K. oxytoca*, they speculated that cell extracts prepared by other procedures would be shown to contain an acetoin racemase activity.

Acetoin Reductase

Scientists reported the purification and characterization of diacetyl (acetoin) reductase from *K. oxytoca*, which catalyzed the reduction of acetoin to 2,3-BD and the reduction of diacetyl to acetoin (Scheme 1).



Scheme 1

Acetoin reductase is a tetramer with a molecular weight of 100 000. Upon isoelectric focussing of an apparently homogeneous preparation, at least 12 species all possessing enzymatic activity with respect to acetoin were detected. Kinetic constants were determined using commercially available acetoin and 2,3-BD and are summarized in Table 7.3.

Table 7.3 : Kinetic constants for Acetoin (Diacetyl) Reductase

Constant	Value (μM)
K_m (NADH)	9
K_m (NAD ⁺)	180
K_m (acetoin)	530
K_m (2,3-BD)	11 300
K_i (NADH)	11
K_i (NAD ⁺)	140

* Phosphate buffer, pH 7.0, 35 °C (Larsen and Stormer, 1973)

The reduction of acetoin following in an order sequential Bi-Bi mechanism

Kinetics of 2,3-Butanediol Formation from Acetoin

The results workers have been extended by others to formulate a kinetic model based on studies using cell-free extracts from *K. oxytoca* grown on D-xylose, as well as acetoin reductases (E₁ and E₂) isolated from the extracts. The cell-free extract was found to have acetoin reductase activity. Based on their activities with respect to

D-(-) and racemic acetoin, E_1 and E_2 were found to be *meso*-2,3-butanediol : NAD^+ oxidoreductase (L-(+)-acetoin forming), and L-(+)-2,3-butanediol: NAD^+ oxidoreductase (L- (+)-acetoin forming), respectively.

The kinetic model which describes the activities of these enzymes is shown in Figure 7.6. The major difference between this model and the one of others (Figure 7.5) lies in the stereo-specificity of acetoin reductases.

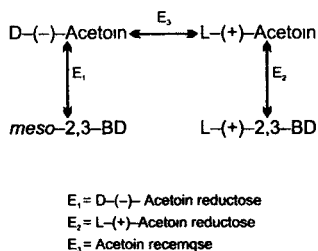


Fig. 7.6 : Modified mechanism for formation of 2,3-butanediol stereoisomers based on studies with enzyme activities isolated from *K. oxytoca* enzyme preparation

The properties and kinetics of D-(-)- and L-(+)-acetoin reductases have been determined. These reductases exhibit an ordered Bi-Bi mechanism.

The reaction equation for D-(-)- acetoin reductase is:

$$v = \frac{V_f V_i ([A][B] - ([P][Q]/K_{app}))}{DEN} \quad (1)$$

where

$$\begin{aligned}
 DEN = & V_f K_{iA} K_{mB} + V_f K_{mB} (A) + V_f K_{mA} (B) + \frac{V_f K_{mQ} (P)}{K_{app}} \\
 & + \frac{V_f K_{mP} (Q)}{K_{app}} + V_i (B) (A) + \frac{V_f K_{mQ} (A) (P)}{K_{app} K_{iA}} + \frac{V_i (P) (Q)}{K_{app}} \\
 & + \frac{V_f K_{mA} (B) (Q)}{K_{iQ}} + \frac{V_f K_{mB} (P) (Q)}{K_{B} K_{app}} + \frac{V_i (B) (A) (P)}{K_{SP}} \quad (2)
 \end{aligned}$$

(A) = (NADH)

(B) = (D-(-)-acetoin)

(P) = (*meso*-2,3-BD)

$$(Q) = (NAD^+)$$

$$v = \text{reaction velocity}$$

$$K_{app} = \left(\frac{(P)(Q)}{(A)(B)} \right)_{eq}$$

The constants are defined in Table 7.4.

In the absence of products, equation (1) reduces to:

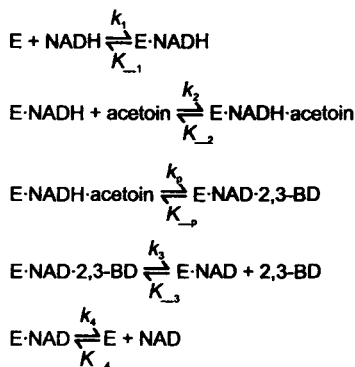
$$v = \frac{V_i(A)(B)}{K_A K_{mB} + K_{mB}(A) + K_{mA}(B) + (A)(B)} \quad (3)$$

where v is the reaction velocity and the other parameters are defined in Table 7.4. This equation represents the rate which would be expected during the initial period of reaction when product accumulation is small.

Table 7.4 : Definition of Constants for the Ordered Bi-Bi Mechanism (Segal, 1975)

K_{mA}	$k_3 k_4 / k_1 (k_3 + k_4)$
K_{mB}	$k_4 (k_{-2} + k_3) / k_2 (k_3 + k_4)$
K_A	k_{-1} / k_1
K_B	$(k_{-1} + k_{-2}) / k_2$
K_{mP}	$K_{-1} (k_{-2} + k_3) / k_{-3} (k_{-1} + k_{-2})$
K_{mQ}	$k_{-1} k_{-2} / k_{-4} (k_{-1} + k_{-2})$
K_P	$(k_3 + k_4) / k_{-3}$
K_Q	K_4 / k_{-4}
V_i	$k_3 k_4 (E) / (k_3 + k_4)$
V_r	$k_{-1} K_{-2} (E) / (k_{-1} + k_{-2})$

Constants refer to reaction sequence given in the general reaction sequences:



where the k 's are kinetic constants, E_i = total enzyme, and stereo configurations given in Fig. 7.5

Values of these constants based on initial rate studies for E_1 are given in Table 7.5. The apparent equilibrium constant may be calculated by using a Haldane relationship.

$$K_{app} = \frac{V_i K_Q K_{mp}}{V_r K_A K_{mB}} \quad (4)$$

Table 7.5 : Kinetic Constants for Acetoin Reductases

Constant*	E_1 D-(-)-Acetoin reductase value	E_2 L-(+)-Acetoin reductase value
K_{mA}	7.4 μM	—
K_{mB}	460 μM	—
K_{A1}	10 μM	17 μM
K_B	3900 μM	—
K_{mp}	2200 μM	—
K_{mQ}	56 μM	—
K_Q	150 μM	20 μM
K_p	29000 μM	—
V_i	0.59 IU μg^{-1} protein	0.016 IU 17 μg^{-1} protein
V_r	0.41 IU μg^{-1} protein	—
K_B^b	—	6600 μM

* A = NADH. B = D-(-)-acetoin, = *meso*-2,3-BD.

Q = NAD⁺. 1 IU = formation of 1 μmol of product at 30°C, pH 7.5. ^bB = L-(+)-acetoin.

Substituting the values for the constants given in Table 7.5 in equation (4) yield $K_{app} = 103$. The K_{app} determined experimentally has a similar value of 113. Hence, this shows that the kinetic constants obtained are consistent with the experimental data.

The data indicated that the enzymatic reduction of D-(-)-acetoin is essentially irreversible (i.e. $k_2 = k_p = k_3 \simeq 0$ in Table 7.4). A similar observation was made for L-(+)-acetoin. Hence, an integrated rate approach was used to determine the key kinetic constants for L-(+)-acetoin reductase. These values are also given in Table 7.5 (see E_2).

The magnitudes of the constants are similar to those reported (Table 7.3).

Analysis of Butanediol by Liquid Chromatography

Modeling of the reduction of the stereoisomers of acetoin to the stereoisomers of 2,3-BD requires an analytical tool which allows the separation of at least some of the stereoisomers. A liquid chromatography technique has been reported which resolves *meso*- from L- and/or D-2,3-BD, from diacetyl or acetoin, and from their precursor sugar, xylose. This technique permits precise quantification without prior sample work-up and is suitable for preparation scale procedures.

The technique consists of injecting a 10 to 100 μ l sample and eluting it with water through a 6mm ID \times 60 cm length column packed with Aminex 50W \times 4 (Biorad Laboratories, Richmond, CA) at 85°C. A typical chromatogram is shown in Fig. 7.7.

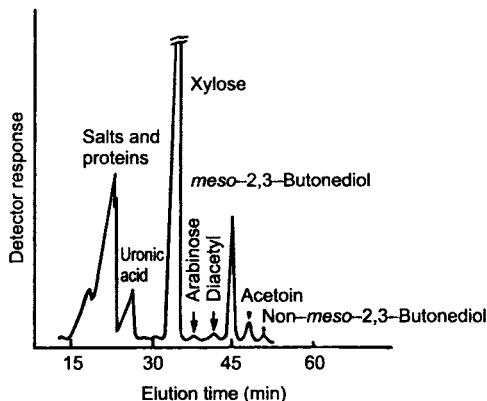


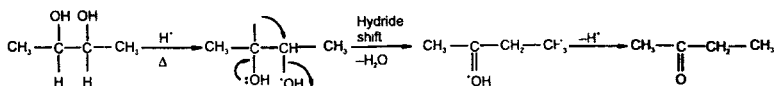
Fig. 7.7 : Liquid chromatogram of a 31 fermentation broth of D-xylose by *K. oxytoca*, after 8h.

Chemistry

There are several interesting chemical reactions of 2,3-butanediol. These include dehydration to methyl ethyl ketone (industrial solvent), reaction with acetone to produce a 'tetramethyl' compound (a possible gasoline blending agent), and formation of butene and butadiene.

Preparation of Methyl Ethyl Ketone (MEK)

MEK can be produced by dehydration of 2,3-butanediol. The dehydration can be carried out using catalysts such as alumina or by direct reaction with sulfuric acid. The reaction mechanism involves a hydride shift (Scheme 2).

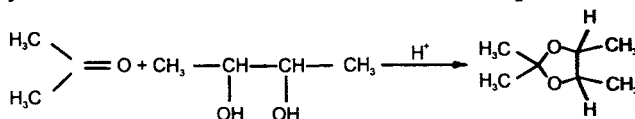


Scheme 2

MEK is an industrial solvent and may find use as a liquid fuel additive.

Tetramethyl Compound

The tetramethyl ether may find use as a blending agent for gasoline, similar to MTBE (methyl *t*-butyl ether). In fact, it has been pointed out that synthesis of MTBE and other new alkyl ether blending agents from sources other than petroleum stocks is essential if they are to be of much real benefit in extending gasoline supply. Both acetone and 2,3-BD are fermentation products.

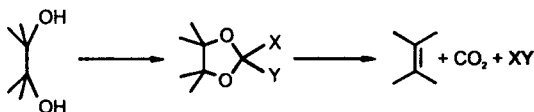


Scheme 3

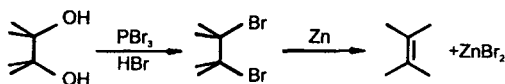
Preparation of 2-Butene and 1,3-Butadiene

Several reductive elimination reactions have been described in the literature which allow the conversion of a 1,2-diol into the corresponding alkene by the breakdown of the intermediate 1,3-dioxolane according to Scheme 4. These reactions proceed with a high degree of *syn* stereospecificity and can be readily applied to the preparation of *cis* and *trans* isomers of but-2-ene from 2,3-butanediol.

Treatment of the diol with PBr_3/HBr , followed by Zn powder, should also result in the formation of 2-butenes (Scheme 5). The debrominations also proceed with a high degree of *anti* stereospecificity, the *meso* isomer giving the *trans*-butene, and the (\pm) isomer the *cis* butene.



Scheme 4

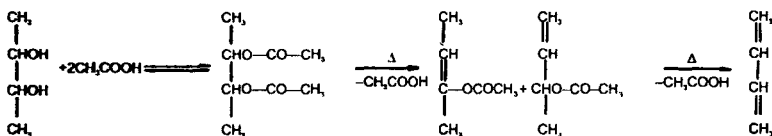


Scheme 5

The butenes can be catalytically dehydrogenated to 1,3-butadiene in the presence of superheated steam as a diluent and a heating medium.

Butadiene can also be obtained in good yield by the direct dehydration of 2,3-butanediol over thoria catalyst, although most other dehydration catalysts give methyl ethyl ketone as the main product.

Earlier work reported the esterification of 2,3-butanediol with acetic acid followed by pyrolysis of the diacetate to butadiene (Scheme 6).

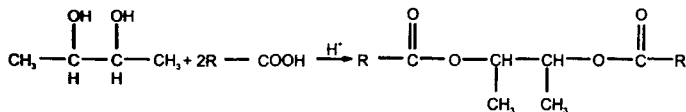


Scheme 6

Butene and butadiene are important industrial chemicals and are currently obtained from cracked petroleum.

Plasticizers

The esters of butanediol and suitable monobasic acids could find use effective plasticizers for thermoplastic polymers such as cellulose nitrate, cellulose nitrate, cellulose triacetate, cellulose acetate butyrate, polyvinyl chloride, polyvinyl esters, polyacrylates and polymethylacrylates. The diesters can be prepared by the usual esterification reactions with monobasic acids or their functional equivalents (Scheme 7).



Scheme 7

Conclusions

2,3-Butanediol is an example of potential bulk chemical which can be produced by fermentation. While a process (Fig 7.8 and 7.9)

for 2,3-BD recovery has been piloted, enhanced efficiency, both in energy consumption and product recovery, will aid the scale-up of the laboratory fermentations.

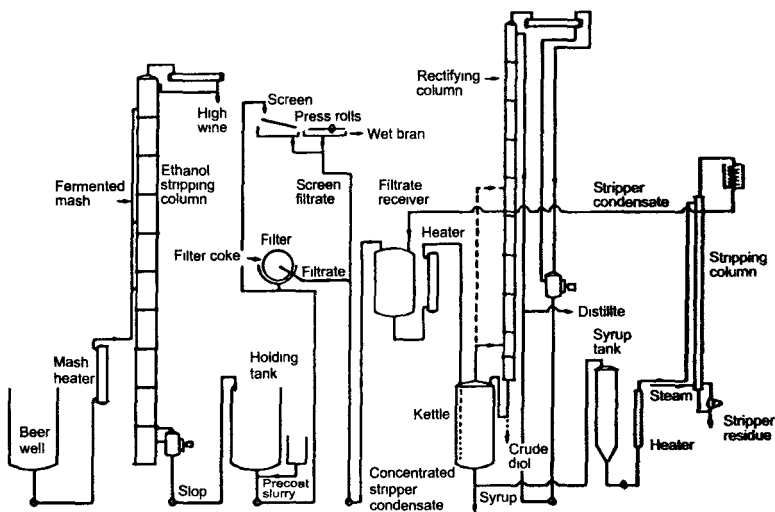


Fig. 7.8 : Pilot plant flowsheet (from Wheat *et al.*, 1948) for 2,3-BD recovery from fermentation beer.

Meanwhile, further laboratory studies are warranted, for example, the development of strains of bacteria which are able to produce 2,3-BD from solutions containing high total solute concentration (derived from hydrolysis of biomass) at the same rate as from laboratory-prepared sugar solutions, and the development of more efficient bioreactors which will maximize conversion rate while minimizing the net production of cell mass. There is also need for further understanding of the kinetic behavior of the different enzymes involved in the biochemical pathway. The use of new analytical techniques has opened the way to re-evaluation of models existent in the literature.

The improvement of our knowledge of the 2,3-BD fermentation will not only enhance the chance of its commercial use but will also add to our general knowledge of bacterial fermentation.

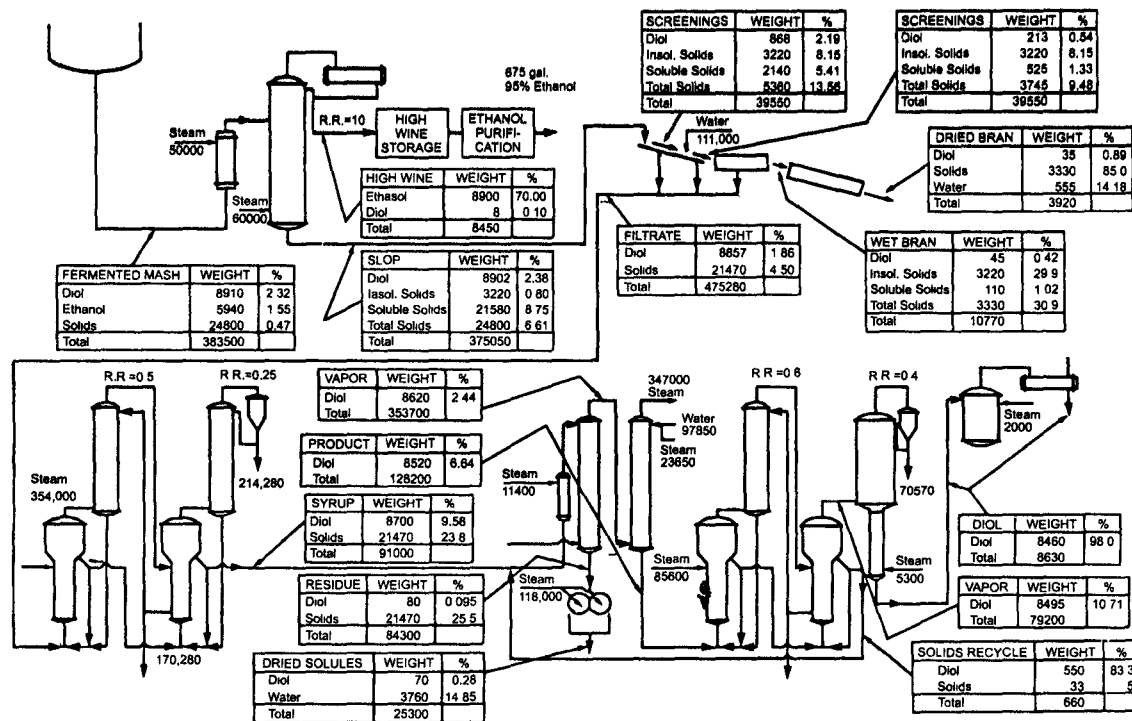


Fig. 7.9 : Material balance for commercial flowsheet for the production of (-) 2,3-BD based on 1000 bu. of wheat per day.

8

Olives

The table olive is the fruit of the varieties of the cultivated olive tree. *Olea europaea sativa Hoffo. Link.* The natural bitterness of the fruit can be eliminated, at least in part, by processing, and it then becomes a fruit whose taste makes it suitable for human consumption, either as an appetizer or a nutrient.

We find the first reference to the existence of this tree in the Holy Bible, where in Genesis the flight of the pigeon with the olive branch is described, announcing the end of the flood.

The traditional methods of processing and the technologies derived from them as well as the classification of the fruit are basically concerned with the ripeness of the fruit and with the color of the final product. Four fundamental types have been established: Green, turning color, natural black, and black. The first three types refer to the color of the fruit as a raw material: a color which does not change during processing. The last type black, is known in California as maduras (ripe) or negras maduras (black ripe) and comprises fruit which is harvested during the stage of turning color, and which is blackened by oxidation in an alkaline medium.

Bitterness of the fruit can be eliminated completely and quickly by alkaline hydrolysis, that is, by treatment of the olives with sodium hydroxide solutions prior to the fermentation and by storage in brine or in dry salt. These fruits are generally called pickled olives. Bitterness may also be eliminated slowly and partially without prior alkaline treatment during the acid fermentation by placing the fruit directly into brine or by preserving it with dry salt. These types are generally known as *olives in brine* or *olives in dry salt*. Variations of these processes according to regional or national custom have resulted in products of varied importance and commercial distribution on an international scale.

Only the three most widely distributed and investigated types will be considered:

- Spanish style pickled green olives in brine
- Natural black olives in brine
- Pickled black olives in brine.

For the Spanish style pickled green olives the lactic acid fermentation is fundamental. For the natural black olives in brine lactobacilli may or may not be present. Other microorganisms, especially yeasts which tolerate high concentration of sodium chloride, predominate and lactic acid bacteria constitute a minor part of the total population. Finally, for black olives pickled in brine, the fermentation process is only important during the previous of storage in brine, prior to processing.

Composition of the Fruit

Fresh Fruit (Raw Material)

The olive is a meaty stone fruit. It is intensely bitter. Its shape is roundish and generally elongated. During ripening the color changes from green to purple or red. Its total weight may vary between 0.5 to 20 g but generally falls within the range of 3 to 10 g. The length of the fruit is usually between 2 and 3 cm and its transverse diameter between 1 and 2 cm. The specific weight is close to unity. The pulp accounts for 70 to 90 % of the weight of the fruit and the endocarp accounts for the remaining 10 to 30%. The seed weighs less than 10% of the weight of the stone.

Many different varieties of olive trees are grown in various countries for the processing of olives. In some case the trees are indigenous and in others the trees have been introduced from other regions. In the latter case they may have undergone evolutionary changes and may have acquired different characteristics.

The above mentioned physical characteristics of the fruit as well as the chemical composition of the pulp depend on several factors. Among these the variety and the state of maturity or ripeness at the time of harvest are the most important. To a lesser extent variations in these characteristics depend on the geographical location, the quality of the soil, and the type of cultivation on irrigated land or on dry non-irrigated but arable land.

The composition of the fruit and the fresh pulp is shown in Table 8.1 with special attention to these chemical compounds which are important for the fermentation and the conservation in brine.

Table 8.1 : The Composition of the Fruit of the Olive Tree and of the Fresh Pulp

<i>Fruit</i>			<i>Weight %</i>
Pericarp	Epicarp Mesocarp	Pulp	70-90
Endocarp		Stone Seed	9-27 1-3
<i>Pulp</i>			
Moisture			50-75
Lipids (oil)			6-30
Reducing sugars, soluble			2-6
Non-reducing sugars, soluble			0.1-0.3
Crude protein (N \times 6.25)			1-3
Fiber			1-4
Ash			0.6-1
Other components			6-10

The major soluble sugars in the order of decreasing importance are glucose, fructose and to a minor degree sucrose. Small concentration of xylose and rhamnose are also present. The presence of mannitol, in the approximate range of 0.5-1% of the weight of the fresh pulp, must be emphasized.

The content of phenolic components is between 1 and 3% of the weight of the ripe, fresh pulp expressed as tannic acid. The bitter factor known as oleuropein has only recently been determined by measurement of its adsorbancy at 345 nm. Catechol oxidase is the enzyme responsible for the change of color of the fruit from green to brown. It has been studied in detail during the ripening of the fruit.

Organic acids and their salts are present in the juice of the fruits in concentration between 0.5 and 1% based on the weight of the pulp. They are of importance because of their buffering capacity of the fermented product.

Final Product

Until quite recently few facts were known about the composition of the final product. All of these concerned the gross composition such as moisture, oil, protein, residual soluble sugars, total carbohydrates, ash, fiber, and organic acids produced during the fermentation, as well as a few data on certain vitamins such as vitamin A, thiamin and riboflavin.

Table 8.2 : The composition of Green Olives pickled in Brine, Spanish Style, Concentration Ranges in the Pulp

<i>Component</i>	<i>Concentration Range (weight %)</i>
Moisture	61.00-80.56
Lipids	9.05-28.19
Protein	1.00-1.45
Fiber	1.40-2.06
Ash	4.19-5.46
<i>Vitamins</i>	<i>Concentration Range</i>
Carotene	0.02-0.23 mg/100 g
Vitamin C	1.44-2.87 mg/100 g
Thiamin	0.40-3.37 µg/100 g
<i>Essential</i>	<i>Concentration Range</i>
<i>Amino Acids</i>	<i>(mg/100 g)</i>
Valine	55-157
Isoleucine	43-121
Leucine	82-227
Threonine	5-64
Methionine	13-79
Phenylalanine	39-111
Lysine	5-31
Tryptophan	13-18
<i>Minerals</i>	<i>Concentration Range (mg/100 g)</i>
Phosphorus	7-21
Potassium	34-109
Calcium	35-86
Magnesium	6-40
Sodium	1313-1753
Sulfur	14-38
Iron	0.58-1.16
Manganese	0.06-0.12
Zinc	0.25-0.41
Copper	0.42-0.82
<i>Caloric Value</i>	<i>Calories per 100 g</i>
	102-280

Spanish style pickled green olives have recently started to receive attention Table 2 shows the concentration ranges for some Spanish varieties. Other essential amino acids such as arginine and histidine, as well as cysteine (sometimes considered essential) have not been determined by these authors, but have been found in fresh fruit by other authors variable and their concentration depends principally on that the raw material used for processing the variety of olives used as raw material. To a lesser extent it also depends on maturity of the fruit and on processing conditions. During processing the percentage of moisture tends to increase and that of oil remains practically unchanged. The concentration of proteins is slightly reduced probably due to solubilization during the lye treatment and brining during the fermentation. Fiber shows a similar trend.

The percentage of ash increases noticeably, a logical consequence of the alkaline treatment, the fermentation and preservation in brine. There are no significant differences in the protein, fiber or ash content between varieties of olives.

Practically all important amino acids are present in the final product. The concentrations of leucine, aspartic acid and glutamic acids are high. There are appreciable quantities of provitamin A, vitamin C and thiamin, as well of mineral elements, particularly calcium and magnesium.

The caloric value is quite variable since it depends mainly on the lipid content of various varieties.

In conclusion it can be said that the oil fraction is of good quality because of its low concentration of saturated fatty (12-19 %) acids and the presence of 5-8% linoleic acid. The fiber is well balanced. With a contribution of essential amino acids olives processed in this manner provide good food value.

Soluble sugars are the energy and carbon source for the fermentation. They disappear gradually during the fermentation and are absent from the final product. The same is true for the bitter factor, oleuropein, which is mostly hydrolyzed during the preceding alkaline treatment. Organic acids and their salts account for about 1.5 % of the weight of the pulp of the final product. They consist in part of the organic acids originally present in the fruit and in part of those elaborated during the fermentation.

Natural black olives in brine. The contents of water is lower and that of oil is higher than for the spanish style green olives. This is the direct consequence of the state of maturity of the fruit at harvest. It does not vary greatly with the variety

The concentration of soluble sugars which is between 1 and 3 % in the fresh fruit remains relatively high (up to 0.3 % or more) even after a long period of conservation in brine because the fermentation process of the traditional industry is quite slow. The same is true of the bitter factor, which sometimes does not disappear completely when there has been no prior alkaline treatment that hydrolyzes this factor. The tannin content which is 1-2 % in the fruit at harvest remains relatively high for a long period of time. Both components give the final product organoleptic properties which are very much appreciated by the consumer. In the typically alcoholic fermentation the following aromatic components contribute to the organoleptic properties: ethanol (96-99% of the total volatiles), followed by acetaldehyde and ethyl acetate.

Finally, the total acidity (both free and combined) is lower than in green pickled olives, namely between 0.7 and 1% based on the weight of the pulp.

Black olives pickled in brine. The main differences between this product and those previously described are due to the oxidation in an alkaline medium. They are as follows. During conservation in brine the characteristics of the fruit are quite similar to those processed as natural black olives in brine. The only difference due to the state of ripeness are the changing color instead of the black color. In the final product the contents of tannins (0.1-0.2%), of oleuropein (absorbency at 345 nm from 0.01-0.05) of the total acidity combined in the form of salts (0.2-0.3%) are very low (all values based on the weight of the pulp). This is the result of the processing conditions.

Chemical and Biochemical Changes and Final Product Characteristics

A series of changes occurs during processing which can be divided into three categories: a) Compounds which are simply lost during the various phases and operations: b) compounds which are transformed by simple chemical reaction or by the action of enzymes originally present in the fruit or produced by

Table 8.3a : Processing and Compositional Changes of Spanish Style Green Olives Pickled in Brine

<i>Starting Phase</i>		<i>Starting Phase</i>		<i>Starting Phase</i>		<i>General Characteristics of the Final Product</i>
<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	
Alkaline treatment and washing with water	Hydrolysis of oleuropein	Fermentation in brine (mainly lactic)	Lactic acid formation from sugars and other fermentable compounds	Conservation in brine	None under normal conditions	Free acidity about 1% as lactic acid
	Loss of sugars and organic acids					pH approx. 3.6-4.2
	Formation of organic acids from sugars	Secondary action of other microbes	Formation of organic acids	Bottling		NaCl : approx 2-8% depending on commercial process

Table 8.3b : Processing and Compositional Changes of Natural Black Olives in Brine

<i>Starting Phase</i>		<i>Starting Phase</i>		<i>Starting Phase</i>		<i>General Characteristics of the Final Product</i>
<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	
None	None	Spontaneous fermentation in brine	Slow loss of sugars, tannins and bitterness	Conservation in brine	None under normal conditions	Free acidity : about 0.3-0.5% as lactic acid pH about 4.3-4.5 NaCl: 6-10% depending on commercial practice
		Yeasts predominate; lactic acid bacteria sometimes present	Formation of organic acids, ethanol, acetaldehyde and ethyl acetate	Bottling		

Table 8.3c : Processing and Compositional Changes of Black Olives Pickled in Brine (alkaline oxidation)

<i>Starting Phase</i>		<i>Starting Phase</i>		<i>Starting Phase</i>		<i>General Characteristics of the Final Product</i>
<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	<i>Process</i>	<i>Change in Composition</i>	
Fermentation in brine by lactic acid bacteria and yeasts	Slow loss of sugars, tannins and bitterness	Alkaline treatment	Hydrolysis of oleuropein	None	None under normal conditions	pH about 5.8-8.0 NaCl: 1-5% depending on commercial process
	Formation of organic acids, and possibly ethanol and other aromatic compounds	Washing	Loss of sugars and organic acids			
		Oxidation by air				
		Brining	Organic acid formation from sugars			
		Bottling				
		Heat sterilization				

microorganisms: c) compounds produced during the fermentation by different microorganism. Corresponding processes and compositional changes as well as final product characteristics for the three types of olives are summarized in Table 8.3.

Principal Microorganism Involved in the Fermentation Process and the Storage of Olives

As shown in Table 8.3 the main phase of the processing of Spanish style pickled green olives in brine is the lactic acid fermentation which follows the alkaline treatment and the washing. The washing removes the greater part of the sodium hydroxide retained by the fruit. It is desirable to obtain a lactic acid fermentation by homofermentative bacteria which produce lactic acid as the only major acid. This results in a product appreciated by consumers.

Workers also established three stages for the traditional system of fermentation in wooden containers holding 440 kg of olives. These stages differ somewhat from those mentioned above. In the first stage which generally lasts from 48 to 72 hours the brine has initially a high pH which decreases to a value of approximately 6. Some colonies of molds, yeasts, sporulating Gram negative bacteria of the coli-aerogenes type develop. At the end of this stage lactic acid bacteria appear. During the second stage (up to 10-15 days) lactobacilli and yeasts generally develop quickly once a pH of 6 has been reached, and the Gram negative bacteria decrease until they disappear completely. Sporulating Gram positive bacteria disappear from the 4th to 5th day on.

During the third stage, which lasts until the fermentable substrates are exhausted, neither Gram negative nor sporulating Gram positive bacilli can be found. Only species of *Lactobacillus* abound and co-exists with a yeast flora. The dominant species is *L. plantarum* and to a much lesser extent *L. delbrueckii* which is also homofermentative.

Lactobacilli start their development during the first 8-13 days. Their maximum growth is approximately between 19-30 days, and they remain viable until the end of the fermentation. Few yeasts can be seen throughout the entire fermentation.

Finally, it is important to note that an increase in volatile acidity (acetic and propionic acids) can occur at the end of lactic acid

fermentation when the fermentable substrate has been exhausted. This may occur during conservation in large containers at the expense of lactic acid, exclusively or almost exclusively. It is followed by a rise of the pH and can lead to undesirable changes in the fruit. This can be called the 4th stage of the fermentation. It is due to species of *Propioni bacterium* which are relatively tolerant to higher salt concentrations. Suppression of these organisms requires at least 8% salt at normal pH values between 3.7 and 4.0 at the end of the fermentation.

All of these circumstances account for a very variable acidity which remains usually within moderate limits of 0.1 to 0.6% expressed as lactic acid. Under some circumstances when the concentration of salt is low enough a population of lactobacilli predominates clearly. It may produce enough acidity and lower the pH sufficiently to change the natural black color of the fruit considerably. During the first days of the fermentation a population of Gram neagative bacteria develops. These organisms gradually disappear when lactic acid bacteria and yeasts become predominant of microorganisms during the first stage of the processing of black olives pickled in brine is quite similar. The same is true for other process for olives in brine, which have not been treated with alkali before being placed into bring.

Description of the Major Processing Methods

A. Spanish Style Pickled Green Olives in Brine

This is without doubt the most important process for the international market. The fruits are harvested when their color is still yellow or a greenish yellow. They are treated with a dilute solution of sodium hydroxyde between 1.3 and 2.6% (weight / volume) for a certain time, normally between 6 and 10 hours. This eliminates the greater part of the bitter oleuropein. In this treatment the lye penetrates the pulp to a depth of tow third to three fourth of the distance between the skin and the stone. The fruits are then washed with water for variable time periods to eliminate the major portion of the lye. They are then placed into a brine of sodium chloride of 9 to 11 Be in which the lactic acid fermentation takes place.

After the fermentable substrate is exhausted the fruits are kept in the same brine until they are packaged and sold. They are sold

in bulk in plastic containers holding 150 to 300 kg of fruit using the same brine in which they were fermented or a freshly prepared brine or a mixture of both. For hermetically sealed consumer packages a freshly prepared brine is used. These consumer containers may be heat-pasteurized.

Prior to bottling the stones of the fruit are usually removed mechanically, and the olives are stuffed with various edible foods.

Harvesting is still done by hand although numerous attempts have been made to harvest mechanically. Tree shakers and other mechanical contrivances have not been accepted because of damage to the trees. The fresh fruit is generally transported to the factory in crates of perforated plastic which allows access of air. The capacity of the crates is from 20 to 25 kg, and the fruit remains in them for a period varying from several hours to 3 or 4 days depending on the variety. They are then subjected to treatment with lye solution.

The containers used for the further processing-treatment with lye, washing, fermentation, storage in brine are fermentors made of polyester and glass fiber. These can be closed completely to create anaerobic conditions and to exclude the growth of film yeasts. The containers have a large funnel on top and valves which facilitate the unloading of the fruit (if it is done by gravity) and which are useful for circulation of the brine. Such fermenters have a capacity of about 10000 kg of fruit. For fermenters which are underground the funnel is used for all loading and unloading operations, and the brine is recirculated by use of adequate pumps.

The strength of the lye solution and the time of immersion in this solution and the desired penetration may be varied and depend on a number of factors. The most important are variety, maturity of the fruit, and temperature. Good control of these factors is essential for the regulation of the speed, the effectiveness and the yield of the fermentation. But it is equally essential for the organoleptic quality of the final product, principally for its color and texture. During the lye treatment a portion of the sugars is removed and a portion is converted to acetic acid. This contributes to the volatile acidity that is always present to the volatile acidity that is always present in the fermented fruit.

The duration and the number of washings are equally decisive. A large part of the lye that has remained in the fruit as the result of the alkaline treatment and a portion of the sugars are removed. An

excessive number of washings can deplete the fermentable compound so that nutrients have to be added to permit the fermentation to proceed. A long time period of washings can lead to undesirable bacterial contamination before the fruit is placed into brine. This may prolong the first stage of the fermentation. On the other hand excessively short washings lead to the retention of high concentrations of bound organic acids ("residual lye") in the pickled fruit. As we shall see later this prevents the attainment of suitable pH values for adequate conservation of the fermented fruit. A rapid rinse after the alkaline treatment followed by a first washing of 2-3 h and a second washing of 10-20 h is considered adequate.

The concentration of the initial brine is also very important. If the concentration of salt is too low the low osmotic pressure can lead to spoilage by sporulation microorganisms of the clostridial type during the first stage of the fermentation, and the pH may remain too high. If, on the other hand, the concentration of salt is too high the fruit may become wrinkled, sometimes irreversibly. Depending on the variety and the maturity of the fruit the concentration of salt must be regulated within the above mentioned limits.

After the initial decrease of the salt concentration it must be elevated gradually during the fermentation in order to maintain good texture in the fruit and a good fermentation and conservation. But the concentration of salt must never be too high to interfere with the growth of lactobacilli. As an approximation it can be stated that the concentration of salt should be between 5 and 6% during the greater part of the fermentation, rising to 7% at the end of the fermentation. Thereafter it may be increased to 8% or even higher during the period of storage to avoid the growth of *propioni bacterium* during the "fourth stage" and a corresponding depletion of lactic acid.

The main factors or parameters that must be considered with regard to the fermentation are the microbial population, pH, free and combined organic acids, the concentration of sodium chloride and that of soluble sugars.

As indicated above the microbial population may consist mainly of Gram negative bacteria of coliform type during the first stage of the fermentation. If these microorganisms persist or are present in large numbers they may lead to spoilage of the fruit

known as "alambrado" or "fish-eye". This results in the destruction of the texture of the fruit and a change in flavor due to a shift in the ratio of volatile acidity to fixed acidity. The development of such undesirable microorganisms and the serious consequences they cause can be prevented by acidification or the injection of CO_2 into the brine during this stage.

Lactic acid producing cocci may be present during the first and second stage of the fermentation. These cocci provide a good degree of acidity for the conservation of the fruit. However, some of these cocci are heterofermentative with a low yield of lactic acid. That means that a pH of 4 which is most suitable for the conservation of the fruit is not reached in a sufficiently short time. It also means that additional nutrients may have to be added later to stimulate growth of lactobacilli with a rise of operational costs (unpublished data of the author).

All of these considerations suggest that the first and second stage of the fermentation be kept as short as possible. With good sanitation and good initial control of the pH - either by acidification or injection of CO_2 - the population of Gram negative bacteria can be reduced to insignificant levels and spoilage can be prevented. However, an initial population of lactic acid producing cocci may persist and predominate over the lactobacilli for a long time. In all such cases inoculation with pure cultures of *Lactobacillus* with a well fermented brine with a good population of active lactobacilli is necessary and convenient. Therefore, in certain cases it can become necessary to accelerate the start of the third stage.

The concentration of salt for preservation and a decreasing pH during the fermentation are essential for the regulation of the growth and fermentative activity of lactobacilli during the fermentation, and later on for the preservation of the fruit and the prevention of spoilage. It has already been mentioned that the concentration of salt must be raised to 7% at the end of the lactic acid fermentation. Thereafter it must be increased to 8% or higher to prevent growth of *propioni bacterium*. The latter organism metabolizes lactic acid with a consequent rise in pH and the possibility of the development to a bad taste and a characteristic bad odor called "zapateria".

A low pH value and a high acidity together with the salt contribute to the organoleptic character of the product and to its

preservation. But it is important to recognize that both pH and acidity are related to a third factor, combined organic acidity or "residual lye" which remains in the balanced medium, the juice of the pulp brine. Relatively high values of "residual lye" counteract the effect of high acidity, maintaining a pH value too high for good preservation. On the other hand, excessively low values of "residual lye" in the fermented product may lead to reductions or accidental loss of acidity, cause a quick rise in pH and spoilage.

Under normal conditions and taking account of all of the mentioned factors the final product of the fermentation should have a pH value in the neighborhood of 4 (3.8-4.2), a free acidity, expressed as lactic acid and consisting mostly of lactic acid, of 1% (0.8-1.2%) and a combined acidity or residual lye of about 1N (0.09-0.11N approximately). The final concentration of salt should be about 7% if the product is expected to be sold in a short period of time, if the sanitary conditions are normal, and if the temperature is not too high. For longer periods of preservation or for extremely high temperatures it is advisable to raise the salt concentration to 8% or higher. The microbial should consist of lactobacilli in their declining phase and of some fermentative yeasts. The final product bottled in small, hermetically closed containers has the following ranges: pH 3.2-4.1: free acidity 0.4-0.6% as lactic acid: NaCl between 5 and 7% residual lye between 0.02 and 0.07 N. The selection of values within these ranges may be influenced by the fact that the containers are or are not pasteurized. The setting of these values is also important to avoid the formation of sediment.

B. Natural Black Olives in Brine

This process has practiced traditionally in Greece and still has great importance there harvest of the generally starts when the fruit is completely ripe but not overripe, that is, when the skin has a color between violet and black. There is a great difference between fruit picked at the beginning and at the end of the season. The olives harvested early have a good texture but the color after processing is not good. Fruit picked at the end of the season retains excellent color after processing but the texture is less firm. The olives are transported as described for the spanish style green olives. After a first selection at the factory to separate damaged fruit and occasionally after washing to remove superficial dirt they are placed directly into brine with a salt concentration between 8 and 10 Be: in some cases lower, about 6% NaCl.

Concrete tanks are generally used as vessels for the fermentation. They are sometimes partially and sometimes completely underground and hold 10 to 20 tons of fruit. They are lined with paraffin or epoxytype resins. During the past years fermenters of polyester and glass fiber, similar to the ones used for the processing of green olives, have come into use. These are generally buried uderground to avoid the very high temperatures in factories located in hot zones. This is important because fruit that is very ripe cannot be processed at high temperatures. In both types of containers one tries to maintain strict anaerobiosis to avoid the growth of film forming yeasts and surface growth of molds which affect the texture as well as the flavor of the product.

The fermentation process is very slow because diffusion of soluble components through the skin is slow when it has not been treated with alkali (as in the processes discussed above). A complex microbiological population develops during the fermentation. Gram negative bacteria of the coliaerogenes type, Gram positive cocci of lactic acid bacteria, diverse yeasts and species of *Lactobacillus* may be present if the sodium chloride concentration is kept below 6-7%. In the spontaneous fermentations the presence of yeasts is dominant.

If for some reason the dominating organism is a *Lactobacillus*, then the final product will have a low pH (about 4) and the acidity will be higher than 0.6% expressed as lactic acid. This product will have a very different taste from the generally marketed product, and its color will be much lighter resembling that of black natural pickled olives treated with sodium hydroxide.

In most instances, when fermentative yeasts predominate, the final product has pH values between 4.5 and 4.8. The free acidity varies between 0.1 and 0.6% expressed as lactic acid. The low acidity developed and the relatively high pH require a gradual raising of the salt concentration. At the end of the fermentation the concentration of sodium chloride should be 10% or higher.

Once the fruit has fermented it is oxidized by exposure to the air which improves its skin color. They are then selected and classified by size and finally bottled with appropriately conditioned new brine. The containers are either wooden or plastic holding about 130 to 150 kg of fruit, or smaller tinsplate containers holding 10 to 15 kg or plastic bag, acidity expressed as lactic acid is between

0.5 and 0.6% and the salt concentration is between 6 and 9%. These are the most frequent values of commercial products.

C. Pickled Black Olives in Brine (Ripe Olives)

Processing may be carried out by two different production schemes. For the first process the fresh fruit is successively treated with sodium hydroxide solutions for varying periods of time to achieve a progressive penetration of the lye into the pulp. After each alkaline treatment the olives are placed into water and oxidized by injecting under pressure into the water. This oxidation of the polyphenolic compounds permits a complete blackening of the skin of the fruit and a uniform coloration of the pulp.

The number of lye treatments is generally between 3 and 5. Penetration into the fruit is adjusted so that the sodium hydroxide of the first treatment merely passes through the skin. Subsequent treatments are chosen so that they penetrate deeper into the pulp, and so that the lye from the last treatment reaches the stone. The concentration of sodium hydroxide in the lye solution depends on the maturity of the fruit, its variety, the temperature of the treatment, and the desired penetration. It varies between 1 and 2%. The higher concentration usually is used for the first treatment.

For operation of the alkaline treatment, for washing and aeration tanks are arranged in parallels. They may have different shapes and sizes and are made of concrete, stainless steel, polyester, or glass fiber. In all cases they have the same network of pipes for the distribution of pressurized air so that the oxidation process is uniform.

After the lye treatment, the washing and the oxidation the olives are washed several times with water to remove the major portion of sodium hydroxide. Generally, 0.1% of iron gluconate is added to the last wash to stabilize the color achieved by oxidation. The olives are then placed into a brine containing 3% sodium chloride, bottled in glass jars or tin cans, and sterilized by heating. The tinplate used for cans is varnished on the inside.

At the time of harvest the color of the skins of the fruit varies considerably from a yellowish-green to a more or less pronounced purple, even though the pulp may still be light in color. The taste, texture and color of the final product may vary greatly depending on the maturity of the fruit and the variety.

The final bottled product has very different organoleptics properties from the fermented fruits obtained by other processes. The pH values are between 5.8 and 7.9. The sodium chloride concentration is between 1 and 3% in general, though it may be as high as 5% depending on the demand and the taste preference of consumers.

Microbial Insecticides

Introduction and Historical Background

The creatures with which humanity is in conflict, either as a competitor, hunter or victim, are themselves held in abeyance by other species living at their expense. This system of checks and balances may be stable in general, but is not so in detail, and fluctuations of feast and famine can be seen within an overall equilibrium of population and resources.

Until recent historical times virtually all control of mankind's antagonists could be said to be of this 'biological' type, and there was no recourse to an alternative when it proved inadequate. However, the 20th century has seen the widespread usage of chemical pesticides, inventions of human wit for human need. Nearly all agricultural production and a large part of public health projects require their use. Chemical pesticides have been eminently successful, but the drawbacks attendant on their use are now manifest. The two commonest criticisms are that a chemical agent may kill non-target organisms and that the target itself may become resistant to the agent.

If we confine our attention to the control of insect pests it is seen that the enemies that can be marshalled against them fall into two broad classes, first predators and parasites, and secondly pathogenic microorganisms. A dramatic increase in insect numbers in a field or forest is often followed by an increase in insect enemies, macroscopic or microscopic, in turn causing the collapse of such an outbreak population. Observation of this process gives cold comfort to those whose crops have been destroyed: successful practice of biological control depends on application of the agent at such a time or in such amounts that economic damage is minimized.

Conscious attempts to control insect pests by biological means have been made over an extended period of our history. Since those days the number of insecticidal microorganisms available for research has increased tremendously, and records of their field trials are now legion.

Present Use

While the use of predators and parasites for insect control provides a fascinating topic, it falls outside the scope of the present article. Methods of production and application are radically different from those of pathogens, which in this regard have several characteristics in common with chemical insecticides.

Insect pathogens have representatives among the bacteria, fungi, viruses, rickettsiae, protozoa and nematodes. Over a thousand pathogens have been recorded, and many of these could be put to a practical use. Table 9.1 lists the major insect pathogens in use and their producers. By far the greater part is made up of bacteria and viruses, with some fungi.

Table 9.1 : Major Microbial Insecticides in Production

Organism	Target	Characteristic of production	
Bacteria			
<i>Bacillus thuringiensis</i>	Lepidopteran larvae	By fermentation by several firms	Commercial
<i>Bacillus thuringiensis israelensis</i>	Dipteran larvae	By fermentation for WHO	Non-commercial
<i>Bacillus popilliae</i>	Japanese beetle	In collected larvae by Fairfax Biologicals	Commercial
<i>Bacillus sphaericus Fungi</i>	Mosquito larvae	By fermentation by Government agencies	Non-commercial
<i>Beauveria bassiana</i>	Numerous	By fermentation in factories in USSR	Non-commercial
<i>Beauveria tenella</i>	Numerous	By fermentation in factories in USSR	Non-commercial
<i>Metarhizium anisopliae</i>	Numerous	By fermentation in factories in USSR	Non-commercial
<i>Hirsutella thompsonii</i>	Citrus mites	By fermentation by Abbott Laboratories	Commercial
<i>Verticillium lecanii</i>	Glasshouse aphids	By fermentation by Tate and Lyle	Commercial

contd...

Table 9.1 – contd...

Organism	Target	Characteristic of production	
Viruses (forest)			
Sawfly NPV	Sawflies	In collected larvae by Government agencies	Non-commercial
Tussock moth NPV	<i>Hemerocamp spp.</i>	In mass-reared larvae by Government agencies	Non-commercial
Gypsy moth NPV	<i>Porthetria dispar</i>	In mass-reared larvae by Government agencies	Non-commercial
Spruce budworm NPV	<i>Choristoneura fumiferana</i>	In mass-reared larvae by Government agencies	Non-commercial
Siberian silkworm	<i>Dendrolimus sibiricus</i>	Presumably in mass-reared insects in USSR	Non-commercial
Winter moth	<i>Operophtera-brumata</i>	Presumably in mass-reared insects in USSR	Non-commercial
Viruses (agriculture)			
Codling moth GV	<i>Cydia pomonella</i>	In mass-reared larvae by Government agencies	Non-commercial
Potato tuber moth GV	<i>Phthorimaea operculella</i>	In mass-reared larvae by Government agencies	Non-commercial
Cotton bollworm NPV	<i>Heliothis spp.</i>	In mass-reared larvae by Sandoz	Commercial
Cabbage looper NPV	<i>Trichoplusia ni</i>	In mass-reared larvae by Government agencies	Non-commercial
Alfalfa looper NPV	<i>Plusia spp.</i>	In mass-reared larvae by Government agencies	Non-commercial
Cotton leafworm NPV	<i>Spodoptera littoralis</i>	In mass-reared larvae by Government agencies	Non-commercial

Production Methods

The technology used in the production of microbial insecticides varies from that of a cottage industry to that equal in sophistication to the pharmaceutical industry. Present methods can be divided into three classes demanding progressively less labour and more costly equipment. They are: collection and infection, mass rearing and fermentation methods.

Collection and Infection

In cases where a pathogen is highly infectious, or alternatively where protection of an expensive commodity justifies the effort, a control agent may be produced from insects collected from the field. Several sawfly species are economically-important pests in coniferous forests. Their NPV are used in trials or control measures.

Mass Rearing

Mass rearing techniques have been most notably employed in the production and release of radiation-sterilized adults as a means of reducing progeny populations.

Successful production of lepidopterian larvae depends on the availability of a synthetic diet as larvae reared on imported foliage are susceptible to epizootics. Antimicrobials incorporated into diet provide some safeguard against this. Larvae are raised on an agar-based diet, either singly or severally, depending on their cannibalistic proclivities. The diet is inoculated with the requisite pathogen and after it has multiplied in the larvae, cadavers and moribund larvae are collected for processing and formulation. Probably the most complete account of such a procedure is given for the *Heliothis* SNPV virus. Productivity depends on judicious choice of the time of infecting and harvesting larvae and the dose of pathogen given. Although the amount of product obviously varies greatly with the host and pathogen, some degree of standardization has been introduced, for example the number of PIB of NPV recovered from a larva of the cabbage looper *Trichoplusia ni* is commonly considered as 6×10^9 . This is known as a larval equivalent (LE). Mass rearing has two main drawbacks. It is labour intensive, and it is inflexible in that a programme cannot be adapted rapidly to production of another species. Some steps can be taken to alleviate the first problem by automation where possible, e.g. in diet dispensation. The second appears intractable.

At present, attention is focussed on the performance of 'Elcar', but several other viruses can be produced for crop protection by mass rearing. Among likely candidates are the MNPV of *T. ni*, the MNPV of the alfalfa looper *Autographa californica*, and the GV of the codling moth *Cydia pomonella*. Only one nematode pesticide has been produced commercially by mass rearing techniques. This was a preparation of *Romanomermis culicivorax* obtained from *Culex*

pipiens larvae and marketed for mosquito control under the name of 'Skeeterdoom'.

Fermentation Techniques

In general, fermentation offers the best route for the industrialization of insect pathogens. It is possible to produce a diversity of organisms *in vitro* although most efforts have been confined to small-scale laboratory studies.

Nematodes

Members of seven families of nematodes have insecticidal properties and interest in their use is widespread although none has been successfully industrialized. Some non-entomogenous nematodes have been reared in artificial media and so may be amenable to fermenter production. It is not yet known whether any entomogenous nematodes can be induced to reach maturity in deep liquid culture.

Neoaplectana carpocapsae, which attacks about 200 species of insect, can be grown on a variety of solid nutrient media. It is usually produced with its associated bacterium *Achromobacter nematophilus* which apparently supplies nutrients to the nematode. Scientists have investigated the DD-136 strain of *N. carpocapsae* and mass produced on dog food.

A solid substrate such as foam rubber or wood wool was required to allow the adults to mate. The nematodes were cultured in the presence of their bacteria. Complex meat-based media such as homogenized chicken hearts and pig kidneys with beef fat were used. Woakes extended his studies with this type of culture to the production of three *Neoaplectana* and three *Heterorhabditis* species. *Deladenus siricidola* proved highly effective in controlling the woodwasp *Sirex*. The nematode has two life cycles; it parasitizes the woodwasp, but can also feed on a fungus associated with the woodwasp and then give rise to insect-infective individuals. Some was able to culture the fungus on a wheat and water medium and then, on this, rear and harvest the nematode for *Sirex* control.

Fungi

Few entomogenous fungi have been produced on an industrial scale. Those that have include the genera *Beauveria*, *Metarhizium*, *Verticillium*, *Hirsutella* and *Aschersonia*. This should not be taken as

evidence of limited usefulness, rather of the difficulties connected with the development of fungal insecticides. In many respects fungi form the most diverse group of entomopathogens in their cycles, nutrition and targets, and new candidates for insect control are frequently reported.

Production of entomogenous fungi *in vitro* is a complex subject. In some fungi the infectious units are conidia (conidiospores) which are not produced readily in submerged culture. Consequently these agents must be produced on solid or semi-solid media. Infectious spores of *Nomurea rileyi* and *Hirsutella thompsonii* have been produced on a series of agar media of varying nutritional quality, but media based on sterilized cereal grains, corn mash or bran are often used for industrial production. The mechanisms of production vary with choice or with the facilities available. Cultures have been produced in open trays or in sterilized bottles or plastic bags. Horizontally-rotating fermenters have also been used.

Other fungi will form conidia in submerged culture, and infective spore preparations of *B. bassiana*, *Culicinomyces clavosporus* and a single strain of *H. thomsonii* have been produced in this manner.

The nutrition of insect-pathogenic fungi is complex, important in its effect on activity, and as yet poorly understood. Some fungi have not been cultured satisfactorily *in vitro*. A homogenate of larvae of the wax moth *Galleria mellonella* proved most effective. It would appear that many opportunities exist for research into the production and optimization of fungal insecticides.

Bacteria

Insect-pathogenic bacteria have been classified as obligate or facultative pathogens. The so-called obligate pathogens cannot be cultivated successfully *in vitro*, while of the facultative pathogens only those that produce a resistant spore or stable toxic moiety are worth cultivating. Candidates are therefore restricted to the crystalliferous spore former *Bacillus thuringiensis* (B.t.) and its recently discovered variant *B. thuringiensis* var. *israelensis* (H-14) or to the non-crystal producer *B. sphaericus* in which the toxin is in the cell wall. B.t. is a lepidopteran pathogen producing a proteinaceous crystalline toxin (S-endotoxin) which causes paralysis of the larval gut and a thermostable β -exotoxin capable of killing flies and other insects. *B. sphaericus* is a promising pathogen of mosquito larvae, as

is *B. thuringiensis* H-14, which has additional activity against blackfly (*Simuliidae*) larvae.

Media used for B.t. production are complex and are based on such natural products as fishmeal, starch, tryptone and cottonseed flour. Efficacy of the product can vary greatly according to the medium used. Fermenters of up to 12 000 gal capacity have been used. Described conditions favour an optimum temperature of 30°C with aeration. The starting pH is 7.2-7.6. The process is a batch one, being completed in 14-72h depending on how the chosen medium influences sporulation and concomitant crystal toxin production. Refinements of this basic procedure have been reported. Works describe the optimization of a medium for high (4×10^9 spores ml⁻¹) spore crystal production in a chemostat with pH, temperature, aeration, agitation and limiting nutrient (total sugar) concentration for β -exotoxin production in a batch process.

The nutritional requirements for production of toxin of *B. thuringiensis* H-14 are not substantially different from those of B.t. It has been produced in a medium previously used for the culture of B.t, subsp. *kurstaki*.

B. sphaericus is a potentially useful agent for mosquito control in that it is active against several different genera of larvae and persists in the environment. It has been produced on a pilot scale. Its nutrition appears to be undemanding in that its toxicity is similar when it is grown on synthetic, complex or hay infusion media although certain strains may be exceptional.

B. popilliae is a useful agent for the control of the Japanese beetle *P. japonica*. Infection is effected by ingestion of the spores, but infectious spores cannot as yet be produced *in vitro*. However, basing their experimental approach on the observation doubling times (ca. 19 h), grew the bacillus at progressively slower rates in a chemostat. They observed about 1% sporulation after 7 d at slow growth rates (dilution rates below 0.1h⁻¹). It was not stated whether or not these spores were infectious *per se*. It may well be that successful production of spores depends on a critical combination of nutritional and physicochemical factors affecting growth rate.

Viruses

Insect viruses can only be produced in an appreciable quantity by infecting susceptible insects (*in vivo*) or established cell lines (*in*

vitro). It is debatable if the *in vitro* process will ever compete commercially with *in vivo* methods. Nevertheless, a large body of knowledge regarding *in vitro* techniques now exists, and the production of some insect viruses by such means is feasible if not economic.

Workers infected *A. eucalypti* cells with the non-occluded iridescent virus of the beetle *Sericesthis* (SIV). *In vitro* production of occluded viruses took a little longer to realize. Others established a cell line from the fall armyworm *Spodoptera frugiperda* and infected it with its homologous NPV. Most occluded viruses established in cell culture are NPV although a CPV and an EPV have also been reported. Production of complete PIB of GV has now been achieved.

Scientists adapted cells of *A. eucalypti* to growth in suspension culture he provided the basic technique for producing insect viruses in large batch cultures. A great deal of subsequent effort has been expended in optimizing cell growth and virus replication, providing cheaper media and scaling up the process.

Workers describe an optimized roller-bottle technique for production of *A. californica* multiply-embedded NPV (ACMNPV) in *S. frugiperda* cells. Bottles with a surface area of 490 cm² containing 100 ml medium and seeded with 1.25×10^5 cells ml⁻¹ of medium were incubated for 6 d at 26-27.5°C while rotating at 1 rev. 8.5 min⁻¹. At that stage the attached cells had grown to 85% confluency on the bottle surface and were infected with a multiplicity of infection (MOI) of 0.75 virus particles per cell. A further 8 d incubation produced 9×10^9 PIB per bottle. This gave 1.5 larval equivalents (LE= 6×10^9 PIB) per bottle.

Workers noted that PIB production of ACMNPV and *T. ni* (TNMNPV) in lines of *S. littoralis* and cabbage armyworm *Mamestra brassicae* cells could be obtained with equal ease in monolayer or suspension culture and that production in suspension culture was not adversely affected by scale-up. With ACMNPV he obtained concentrations of 1×10^6 PIB ml⁻¹ with *M. brassicae* cells and 2×10^7 PIB ml⁻¹ with *S. littoralis* cells compared to 9×10^7 PIB ml⁻¹ with *S. frugiperda*. Others described the growth of cells of *S. frugiperda*, *S. littoralis* and *M. brassicae* in Biostat V and Microferm (New Brunswick) fermenters of up to 101 capacity for periods of up to 200 d with periodic withdrawal and replenishment of the cell suspension with new medium. In the Biostat fermenters they

obviated the problems of sparging by passing air through a silicone rubber tube coiled inside the vessel. In these studies they employed a medium in which the (normally) most expensive component, foetal bovine serum, was replaced by egg yolk emulsion.

Formulation and Application

Depending on the manner in which they can be handled, insect pathogens can be divided into two groups. Vegetative cells and fungal mycelia often lost viability rapidly. Indeed this may also be true of structures normally thought of as 'resting' or 'resistant', such as some fungal spores. In such cases a normal marketing and distribution system cannot be used. The pathogen must be applied close to its place of manufacture and within a short time.

However, several classes of insect pathogens form relatively durable resting bodies which at least offer the hope of a product having a practicable shelf life of 18 months to 2 years. Among these are bacterial and fungal spores, protozoan cysts and the inclusion bodies of insect viruses. Within certain limits such agents may be formulated in a manner similar to that of chemical insecticides. Suspensions or powders can be produced, and fillers, spreader-stickers and protectants incorporated.

Formulation has, as has application, a critical effect on the efficacy of microbial insecticides.

In the field the factor most likely to inactivate a microbial insecticide is sunlight. Consequently sunlight protectants such as dyes, carbon black or titanium dioxide are added. Workers used protectants in conjunction with microencapsulation with *Hiliothis* SNPV. At present the evidence gathered does not point to a marked or unequivocal benefit conferred by sunlight protectants.

Insect pathogens can be formulated with, or applied at the same time as, some chemical insecticides. Others found that B.t. was compatible with Orthene, Dylox, Sevin, Zectran, Dimilin and Lannate and was incompatible with several others out of a total of 27 insecticides tested. A general effect of chemical-biological combinations is that lower doses of either agent are required for the same result although cases of independent action and antagonism also occur.

However, widespread infestation of cash usually dictates a 'blanket' application of the control agent. Often this is done when

the pest becomes apparent. A 'prophylactic' approach, in which low levels of pathogens are used to control young and emergent larvae, would work in principle provided that the techniques of scouting and forecasting are adequate. Because of their generally restricted host range microbial insecticides are ideally suited to integrated pest control programmes. Arthropod predators and parasites of the pest are left intact to control survivors. This bonus can mean that two or three applications of a pathogen can replace a dozen applications of a chemical spread over the earlier parts of a growing season. Should the system be seen to be failing in the latter part of the season, the pest, along with its enemies, can be attacked with chemical insecticides.

Various methods of application are under study. Microbial pesticides are amenable to conventional ways of dispersal, either as sprays or powders. Pathogens can be delivered as ULV sprays with droplet sizes ranging from 5-30 μm . The effective life of a pathogen can be prolonged by application to the underside of foliage.

Field Performance

The efficacy of microbial control is affected, as are other forms of control to a certain extent, by a multiplicity of factors. Prominent among these are the habitat in which control is to be attempted and the degree of control required, the age and habits of the target, and the weather. The areas in which insect pathogens have been used may be listed as: ponds and streams; forests; stored products; and field crops (subdivided into broad-leaved crops, and topfruit, stems and tubers).

Ponds and Streams

Problems in this area are of either a medical or amenity nature and are usually caused by mosquitos, midges or blackflies. Larvae of these groups are susceptible to several pathogens. The World Health Organization has recommended that several pathogens should be investigated as potential candidates for mosquito control. These include the bacteria *B. sphaericus* and *B. thuringiensis* H-14, the fungi *Coelomomyces*, spp. and *Cliciniomyces clavosporus*, the protozoa *Nosema algerae* and *Vavraia culicis*, and the nematode *Romanomermis culicivorax*. The effectiveness of *B. sphaericus* strain 1593 was confirmed by several field studies, and *B. thuringiensis* H-14 is being developed rapidly for use both against

mosquito and blackfly (*Simuliidae*) larvae. *Coelomomyces* is an effective antimosquito agent as the motile zoospore actively seeks out its host, but the complexity of its life cycle precludes easy manufacture. In contrast Army Medical Corps has mass-produced *C. clavosporus* in a simple medium and demonstrated its efficacy against larvae of mosquitos and some midges (*Chironimidae* and *Ceratopogonidae*).

Forests

In India large stretches of forest are treated with B.t. and viruses. Major pests are the sawflies *Neodiprion sertifer* and *N. leconti* and the spruce budworm *Choristoneura fumiferana* in coniferous forests, and the tussock moths *Hemerocampa* spp., the gypsy moth *Lymantria dispar* and the forest tent caterpillar *Malacasoma disstria* in deciduous forests. In the USSR the Siberian silkmoth *D. sibiricus* and the winter moth *O. brumata* assume importance. From the numerous instances of the use of pathogens in forests some general conclusions can be drawn. As forests can tolerate some defoliation, as opposed to tree killing, control can be less vigorous than that required for cash crops. Viral control of lepidoptera is generally more difficult than that of sawflies, but where this is not feasible B.t. may be used. Both B.t. and viruses have been used in combination with chemical insecticides.

Stored Products

Those occluded viruses which have been found in stored product insects provide a potentially effective means for control. They can be applied at the time of storage, stable conditions aid reproducible performance and the absence of light prolongs activity. Viruses have been used against the almond moth *Cadra cautella*, the Indian meal moth *Plodia interpunctella* and the mediterranean flour moth *Ephistia kuehniella*. Protozoans have been used against beetles of the genera *Tenebrio* and *Tribolium*.

Field Crops

Field crops provide the major market for insecticides. From the point of view of application they can be divided into two categories: broad-leaved crops; and top fruit, stems and tubers. Most work in this area has been done with B.t. and viruses, with a smaller amount of effort being expended on protozoa, nematodes and fungi.

The degree of success reported by authors varies from superiority to chemicals, through comparability with chemicals, to inactivity. Variability in results can be ascribed to a multiplicity of factors which include the intrinsic activity of the agent and how it is handled and applied. In at least one publication an insect pathogen has been deemed inactive when it was used against the wrong pest.

Broad-leaved crops can be protected with relative ease both by chemical and microbial insecticides. When they are applied correctly microbials can exert a degree of control equal or superior to that obtained with chemicals.

Burrowing pests of fruits, stems and tubers are difficult to control by either biological or chemical means as there is only a limited period when the insect is exposed. Normally the pest must be treated at an early age in order to achieve adequate control. Recently workers obtained good protection of apples with the GV of the codling moth *Cydia pomonella*.

As in other areas, B.t. and viruses have been found to be compatible with chemicals when used on field crops. In some cases a synergistic effect may be seen, with lower doses of both agents being required to obtain the same degree of control. Infection with a pathogen such as a subacute polyhedrosis, can increase the susceptibility of an insect to chemical insecticides.

Safety Considerations

In broad terms, authorities charged with environmental protection look favourably on biological methods of pest control. They are regarded as having the twin advantages of lowering the burden of chemical residues on the environment and, in some cases, being effective where the development of resistance has defeated chemical pesticides.

Regulatory authorities do not require safety data to allow the use of predators and parasites for biological control; these are required only in the case of pathogens. Nematodes present an interesting borderline case. In the United States Environmental Protection Agency (EPA) has ruled that *N. carpocapsae* and its attendant bacterium *Xenorhabdus nematophilus* do not require registration.

The specificity of insect pathogens has a direct bearing on the manner in which their safety is investigated. Their toxicology differs from that of chemicals in two respects. Pathogens act by infection,

therefore selected non-target organisms must be examined to demonstrate their immunity to attack, rather than to establish an LD₅₀. In general, where infection does not occur, nolethal dosage can be measured, unless the agent carries a non-specific toxin. In cases where no activity has been shown with non-target organisms, the inference has been drawn that residues of insect pathogens on crops are innocuous. This can lead to some agents being granted exemptions from tolerance as in the case of B.t. spores or the *Hfeliiothis* NPV (Elcar in 1975).

The EPA is committed to reduce the use of chemical pesticides and to promote integrated pest management schemes. To this end it has drawn up interim guidelines for the registration of microbial pesticides and hopes to finalize them shortly. Permits for the experimental use of microbials can be issued after the submission of preliminary safety data. Tests for potential hazards which might occur with certain products are described, e.g. inhalation tests with fungi and allergenicity tests for materials containing insect remains. The guidelines have a several-tier approach. Maximum hazard tests (MHT) are proposed in the first tier. MHT are expected to take into account the worst case that could be encountered, either in size of dosage or in the sensitivity of the route of infection. Tests in subsequent tiers are only required if a potential hazard has been indicated in the first.

Economics

Commercial organizations may produce microbial insecticides for several reasons. They may do so for simple profit, by direct selling or by contract to another firm, but other factors may play a part. Microbials may be produced in spare plant capacity or in order to diversify. From the point of view of the large pesticide manufacturer, microbial insecticides have one major drawback, real or imaginary. Specificity limits market size, resulting in relatively small volume production more comparable to the fermentation than the petrochemical industry. Set against this is the ease with which microbials can now be registered and their low development costs.

Manufacturing costs of microbial insecticides are but poorly documented.

A good microbial insecticide is as cost-effective as a (good) chemical. The potential performance of a microbial is optimal when

the crop is valuable, the environment predictable, the pest susceptible and the delivery system adequate. These conditions are optimized by the glasshouse environment where the cost of the crop and increasing pest resistance to chemicals have stimulated the application of integrated control programmes. This does not mean that performance of microbials under less-regulated conditions is inadequate.

The Future

At present microbial insecticides command but a small proportion of the insecticide market, about 1% of the total in monetary terms. However, they will probably play an increasingly important role in integrated control schemes and in niches where chemical insecticides have lost efficacy or never were effective. Non-commercial organizations and international bodies will continue to play a key part in their development. Although only a small proportion of agents discovered will reach development, microorganisms with novel insecticidal properties are likely to be reported at a steady if not increasing rate.

The cloning and expression of *Bacillus thuringiensis* toxin genes in *Escherichia coli* and *Bacillus subtilis* provide a method of powerful potential for the improvement of this microbial insecticide, by increasing the rate of toxin production or its concentration in the product, or in the spectrum of its targets. The possibility of externalizing a hitherto intracellular product suggests a route for further increasing toxin production many fold.

In many respects the production and use of microbial insecticides is still an infant subject and numerous disciplines can contribute to its growth. There is a pressing need for improvements in formulation and application methods. There is no reason why entomopathogens other than B.t. should not be equally amenable to improvement by equally amenable to improvement by genetic manipulation.

Starter Cultures in Meat Production

Fermented sausage and uncooked ham are fermented raw meat products. Enzymatic activity of certain microorganisms transforms and modifies the raw meat during the process of production. The microorganisms thus are essential ingredients for the fabrication of these foods. A mixture of sterile meat with salt and spices will never yield sausage of this type, nor will salted meat turn into ham without bacterial activity. The interrelationship between meat and the enzymatic activities of bacteria were unknown until fairly recently although people have unconsciously made use of bacteria in food production since ancient times. Only after the discovery of microorganism elaborate scientific investigations permit new developments in the field of food technology. It now became possible to understand the processes of fermentation and maturation of meat products. Through such discoveries man was able to consciously employ microorganisms. It was now possible to influence the processes involved in meatcuring and new curing methods were developed. In fact, the history of food products should largely be understood as a history of the employment of useful microorganism.

Starter Culture Development for Meat Product Technology

Systematic inoculation with microorganisms as it has been in practice in other nutritional sectors such as the brewing and dairy industries was introduced to meat product technology only fairly recently. It nevertheless has been several decades that technologies for unheated meat products are available for use of microorganism in the form of so-called starter cultures. These are single or mixed-cultures of assorted, non-hazardous strains of microorganism. The selected strains carry out specific enzymatic activities to yield specific modifications of the substrate and are used under controlled conditions.

Table 1 includes those microorganism which within the past sixty years have been used in experiments and in commercial production of cured products. Species names are maintained exactly as they appear in the original publications.

Table 10.1 : Microorganisms Used as Starter Cultures for Cured Meat Products

Bacteria	Micrococcus epidermidis Micrococcus conglonteratus Micrococcus candidus
Achromobacter guttatus 921	
Achromobacter 22	Pediococcus cerevisiae
Achromobacter x	Pediococcus cercvisiae NRRL-B-562 Pediococcus cerevisiae CPO 18
Aerobacter cloacae	Pediococcus cerevisiae B-2 Pediococcus specialis PS-23
Acromonas x	Pediococcus acidilactici
Acromonas 19	Pediococcus Pc-30 Pediococcus Pentosaccus
Bacillus subtilis	Proteus vulgaris
Bacillus specialis	
Flavobacterium specialis	Spirilli
Lactobacillus farciminis	Staphylococcus carnosus
Lactobacillus plantarum	Staphylococcus simulans M 111
Lactobacillus P22	Staphylococcus simulans M 17
Lactobacillus NRRL-B-5632	Streptococcus xylosus
Lactobacillus hispanicus CP-9	Streptococcus carnosus
Lactobacillus sake	Streptococcus lactis
Lactobacillus specialis CP-2P	Streptococcus lactis AK 60
Lactobacillus spcialis CP-26	Streptococcus latis var. H.
Lactobacillus lucanicarum B-3	Streptococcus diacetylactis Streptococcus diacetylactis 4284
Lactobacillus cucumeris	
Lactobacillus L 110	Streptomyces griseus
Lactobacillus Pentosus	
Lactobacillus arabinosus	Vibrio costicolus
Lactobacillus leichmannii	Vibrio halo-denitrificans
Lactobacillus pentoaceticus	Vibrio specialis
Lactobacillus acidophilus	
Lactobacillus wehmeri	
Lactobacillus gayoni	Yeasts

contd...

Table 10.1 contd....

Lactobacillus mannitopoeus	
Lactobacillus 4669/6	
Lactobacillus fermentum	Debaryomyces hansenii
Lactobacillus maltaromicus	Debaryomyces klueckeri syn.
	Debaryomyces pfaffii
Micrococcus varians	
Micrococcus aurantiacus M 53	Saccharomyces specialis
Micrococcus specialis MF-1	
Micrococcus specialis M 17	
Micrococcus specialis M 111	
Micrococcus specialis DB6	Modis
Micrococcus aquatis	
Micrococcus P4	
Micrococcus M 104	Penicillium nalgiovense
Micrococcus M 86	Penicillium caseicolum (candidum)
Micrococcus caseolyticus	Penicillium expansum
Micrococcus 199/10	Penicillium simplicissimum

Curing of ham has been recommended with staphylococci, lactobacilli and pediococci. It will be necessary to carry out further basic research in ham curing technology as ham is especially expensive and extraordinarily susceptible to disturbances in the process of fermentation.

Starter cultures have also successfully been used in the production of Binden Meat (Bundnerfleisch). Selected bacterial cultures could be applied in the production of cooked ham and "Kasseler" if sufficient time is allowed for fermentation. Starter cultures have proved effective in pre-curing chunky pieces of meat, for canned sausage and for treating fresh blood. Utilization of lactic acid bacteria as starter cultures for long time preservation of ground meat was also reported.

Meat Curing Technologies

Fermented sausage undergoes a color modification (reddening), it is storable without cooling or refrigeration, and is consumed raw; it spreads easily or may be firm after "ripening" through desiccation. This definition most certainly is also applicable for characterizing fermented sausage throughout the world. Fermented sausage is

normally made from pork and/or beef with bacon (pork). Additives are table salt and saltpeter or nitrite curing salt, sugars, spices and, starter cultures. Further adjuncts and additives are sodium ascorbate and glutamate which are employed for distinct technological reasons. Other additives currently used are either unnecessary from the technological standpoint or may, in fact, be illegal. The utilization of these substances often depends on local or national legislation.

Locally and nationally there are, in fact, some basic differences in the composition of fermented sausage. This makes it difficult to generalize manufacturing processes for fermented sausage. Consequently, it is difficult to make recommendations for fermented sausage production: worldwide there are simply too many variants. Generally we distinguish between easy to spread and firm, fine- and coarse-grained, large- and small-caliber, smoked and air-dried fermented sausage. Every producer has his own concepts, recipes and experiences.

For ham production there is an equally complex variety of products and methods. In general, ham is meat from the posterior extremities of swine. Cured products are also made from parts of the shoulder, from chuck, from rib meat, belly and dorsal bacon. Ham technology knows many different variations. Some products are air-dried, others smoked; there are differences in cuts and in treatment and there are many local specialties. The specific cut of meat to be cured - whether with or without bones - will determine shape and denotation of the product. Thus we distinguish between bone ham, center-cut ham, rolled ham or gammon. Variations of the curing procedures yield additional differences of the product.

Fermented Sausage Production

For fermented sausage a mixture of meat and bacon with spices and additives is fermented to make a fine tasting and esthetically appealing meat product.

Primarily the sausage must have a pleasant appearance. Essential factors are a pleasing color and color stability, and impeccable shape with type-specific, regular granulation, an appealing aroma, a mild taste and firmness, or spreadability. All these qualities are preferably to be achieved within a very short time and as safely as possible. This will guarantee a uniform product which is financially appealing to producer and customer.

Technological procedures consist of:

1. Selection and pretreatment of meat
2. Removal of bones and tendons
3. Cutting, grinding and chopping of meats
4. Mixing the meat
5. Adding adjuncts and additives
6. Filling paste into casings (natural or synthetic) or pressing chain-sausages without casings.
7. Climatic conditioning and "ripening"
8. Smoking or air-drying
9. Maturing and aging

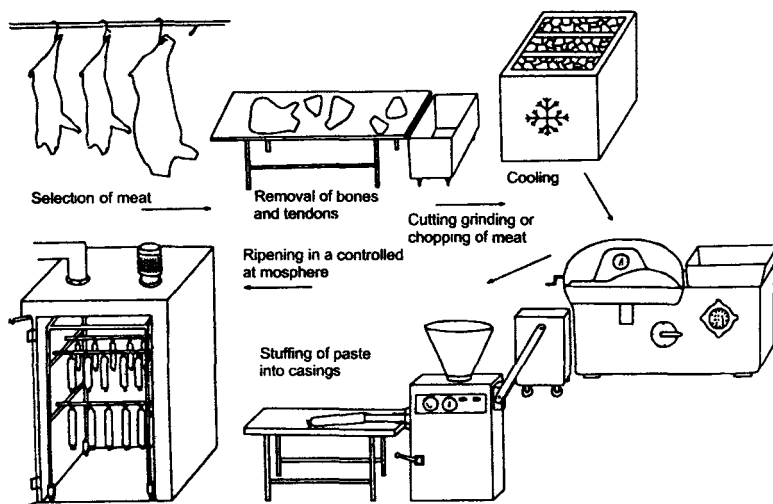


Fig. 10.1 : Flow diagram of the processing of fermented sausage.

The production of fermented sausage and especially the subsequent "ripening" process involve very complex reactions. Technical, chemo-physical and biological processes often occur simultaneously and some reaction are interdependent. Some parameters are measurable, such as the decrease of the pH, changes of redox potential, decreasing water activity, nitrate/nitrite reduction, formation of aroma, degradation of sugars and increase in lactic acid content, activity of meat-borne enzymes and the

fermenting activities of microorganisms. Unfortunately, today only fragmentary scientific knowledge of meat curing processes exists. The more is known about the internal processes, the more we will be able to improve production procedures. We are still far from reaching the point where fermented sausage and ham would come off the conveyor belt in uniform quality despite the perpetual efforts of commerce and industry along these lines.

Ham Production

The production of ham and similar cured products is confronted with a variety of extraordinary handicaps which make it difficult to obtain a uniform quality product. Standardization of the fresh raw meat is especially difficult. Problems associated with the much discussed "dry, firm, dark" (DFD) meat is of significant importance for ham processing. This meat has a dark color, is glutinous and pH values are high. These traits make the meat unsuitable for fresh cured meat production. Often the pH of a load of meat may not have pH values lower than 6.2; mere sorting for low-level pH meat would not yield sufficient starting material for curing. As the entire meat with pH values higher than 6.2-6.4 can not be made into boiled products, it often may be necessary to choose meat for curing which fails to meet the normal requirements. However, correspondence between pH values and suitability for curing still remains a matter of controversy.

The fermentation of cured products can therefore be technically impaired by insufficient quality of starting material. Other insufficiencies may result from improper pretreatment and inadequate cooling and ripening. Through the tendency for shorter curing periods the product often does not obtain optimal structure, appearance, aroma, and taste. It seems logical to employ selected bacteria to overcome the aforementioned difficulties.

Technologies for uncooked ham production differ widely as is exemplified by such national and local specialties.

The multitude of different curing methods is supplemented by further procedures which have locally been adopted through individual experience and tradition. There are three distinct fabrication methods: the dry rub, the cover pickle and the pumping procedures; these being employed either singly or in combination. The main difference between these methods is the varying concentration of table salt, the presence or absence of nitrate or

nitrite and the varying amount of sugar and type of spices. Further distinctions are based on the degree to which the ham is smoked, aged and dried in order to provide final aroma and to ensure shelf life. Drastic differences in processing can be observed for the Italian Parma-style ham, treated with table salt only and subsequently air-dried and on the other hand the Black Forest-style ham, which is cured with table salt, nitrate and juniper-berries and thoroughly smoked until black.

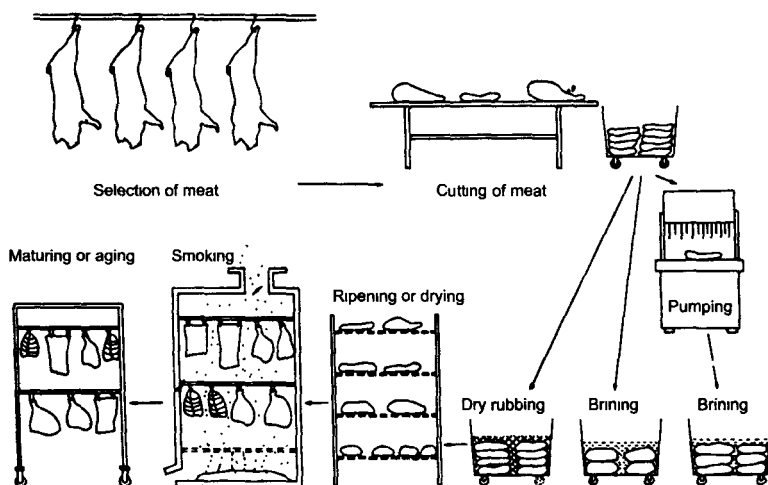


Fig. 10.2 : Flow diagram for the processing of hams.

The procedures in ham production are:

1. Selection and pretreatment of meat
2. Trenching/cutting of meat (with or without bones)
3. Curing
 - a) dry rub procedure
 - b) cover pickle procedure
 - c) pumping procedure
 - d) dry rub followed by cover pickling
 - e) pumping followed by dry rub procedure
4. Ripening
5. Washing and scrubbing

6. Drying
7. Smoking or air-drying
8. Maturing and aging

Temperatures are kept at 4-8°C during the entire process: only the classical smoking procedures require a temperature of 18-20°C. Certainly, these temperatures set a limit to fermentation and the employment of starter cultures. Negative effects on growth and physiological activities of the microorganisms also arise from high table salt concentrations and large sizes of ham. For fermented sausage production temperatures are generally higher (between 18-26°C) and enzymatic activities develop more readily. This most likely is the reason why starter cultures presently are not used to the same extent in ham production as they are in sausage production: in sausage technology low salt concentrations and the ground meat with the larger contact surface facilitate fermentation. Microorganisms play a decisive role in curing processes as they preserve structure and flavor the meat and, therefore, determine the character of the product. Furthermore, undesirable bacteria must be killed during the curing process to preserve the meat from spoilage and make limited storage possible. It therefore make limited storage possible. It therefore is important to combine the "right" bacteria with the "right" techniques which is in fact a challenging task.

Effects of Starter Cultures

The entire ripening process of fermented sausage and ham is quite unthinkable without the enzymatic activity of bacteria. The initial bacterial flora of a sausage- "emulsion" or generally of a piece of meat is extremely variable depending on slaughtering hygiene, transportation and pretreatment of the meat, just to name a few such factors. Predominance of undesirable, detrimental or even pathogenic germs or the absence of critically important bacteria will disturb an appropriate fermentation or will even render fermentation impossible. This often results in quality insufficiencies of cured products. To eliminate these factors starter cultures of selected fermenting microorganisms are employed.

During ripening of fermented sausage two basic microbiological reactions occur simultaneously which influence each other and are directly dependent upon each other:

1. The production of nitric oxide by nitrate and nitrite reducing bacteria.
2. A decrease of the pH of the sausage emulsion via glycolysis by acidogenic microorganisms.

Both reactions are interconnected by two factors: competition of microorganisms for available carbohydrates and the pH dependency of nitrate and nitrite reduction. Nitric oxide formation and pH decrease are the two crucial points of sausage fermentation. A simplified presentation of these interconnections is shown in Fig. 10.3.

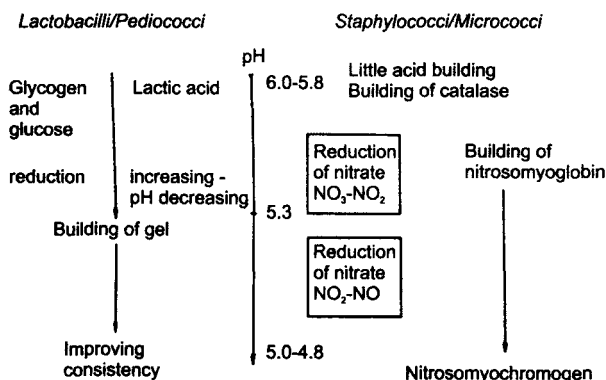


Fig. 10.3 : Effect of starter cultures.

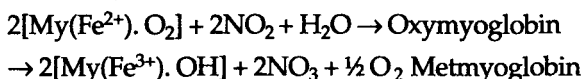
A. Nitrate/Nitrite-Reducing Bacteria

Fresh meat of warm blooded animals obtains its color from its myoglobin content. This compound is formed by a combination of a colorless protein and a colored compound, heme. After slaughtering the myoglobin reacts chemically and turns brown. For esthetical reasons meat products should have an appealing red color. This is accomplished by adding nitrogenous compounds. The formation of red color in cured products results from the inclusion of nitric oxide into the unmodified myoglobin.

When heme binds to the protein moiety the sixth coordination position remains unoccupied. This molecular "gap" may be filled with smaller molecules. One of the most important biological functions is the binding of oxygen. Carbon dioxide and nitrogen can similarly be attached to the macromolecule. Only the

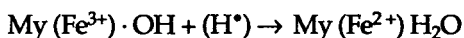
myoglobin with a free binding site at the 6th position is able to adsorb nitrogen; if the gap is filled, nitrogen cannot enter.

In living tissue myoglobin assumes the form of oxymyoglobin. After slaughtering oxygen consuming reactions occurring within the muscles cause a reverse reaction back to myoglobin with its unoccupied coordination site. After cutting the meat the enlarged meat surface once again permits the formation of oxymyoglobin. This is important as oxygen-saturated myoglobin is easily oxidized to the grey-brown metmyoglobin. This reaction occurs spontaneously at room temperature but can be accelerated by oxidizing agents such as nitrite. The following equation is merely a simplification of the otherwise fairly complicated reaction which involves many other compounds:



Metmyoglobin may also adsorb nitric oxide to form the nitrosometmyoglobin.

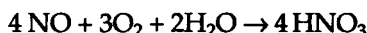
Before the color can turn red oxymyoglobin and metmyoglobin must be reduced to native myoglobin. Reduction of oxymyoglobin is simply through its loss of the oxygen molecule. Metmyoglobin, however, has a trivalent iron ion which must be reduced to the divalent state (H stands for the proton of the reducing agent):



Reduction also leads to the transformation of the red nitrosometmyoglobin to nitrosomyoglobin. Formation of the red color can be accomplished in several ways. The simplest procedure is direct treatment with nitric oxide formed by reduction of nitrates and nitrites. Potassium nitrate and sodium nitrite are mainly used for this purpose. The color can be induced at either high or low temperatures. High temperature procedures operate either very fast (as with canned sausage and cooked ham) or fairly slow (as with fermented sausage and uncooked ham). Within the meat and among the possible additives there are generally more reducing agents which act at high than at low temperatures. Some reducing groups of amino acids of meat protein (e.g. thiol groups as in cysteine and hydroxy groups as in serine) and various sugars, including their catabolic products are high temperature nitrate/ nitrite reducers. Among the reducing additives ascorbic acid is most effective and

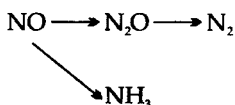
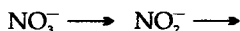
is most widely used for heated curing: it reduces nitrites to nitric oxide and is thereby oxidized to dehydroascorbic acid. The reaction occurs at high rates causing rapid formation of an intense red color.

All chemical reducing agents, including those found within the meat itself have one crucial disadvantage: the meat cannot absorb nitric oxide at the rate at which it is produced. Approximately 50% of the formed nitric oxide does react with the meat however, up to 10% of the nitrite input is transformed via nitric oxide to nitric acid (and nitrates):



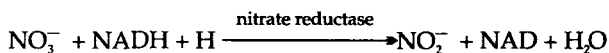
This undesirable nitrate remains in the meat. The amount of nitrates is especially high (more than 10%) if ascorbic acid is used.

Presence of nitrate/nitrite reducing microorganisms is crucial for red color formation in fermented sausage and uncooked ham. Predominantly, these microorganisms are micrococci and staphylococci. They either develop spontaneously in or on the meat or are added as starter cultures. In contrast to the chemical reducing agents the reducing bacteria first transform nitrates into nitrites, then to dinitrogen monoxide and finally to elementary nitrogen. Another pathway leads to the formation of ammonia. In both cases, however, nitric oxide is also formed as a metabolite:



Only under anaerobic conditions can nitrates and nitrites serve as oxidizing agents for bacteria; manufacturers know that only tightly packed sausage will have a uniform color.

Micrococci and staphylococci produce nitrate reductase (EC 1.6.6.1-3) and nitrite reductase (EC 1.6.6.4). These enzymes transfer protons from nicotinamide adenine dinucleotide (NADH) or its phosphate (NADPH) to the substrate:



An advantage of using microorganisms for reduction is their ability to reduce nitrates. They thus remove accumulated nitrates

formed through the side reaction mentioned above. Nitrate reduction by microorganisms must, however, be coordinated with the decreasing pH of the sausage. Very low concentrations of residual nitrates and nitrites can then be obtained by the use of starter cultures. From a practical perspective the amount of added nitrates and nitrites depends on the amount of sugars whose content varies greatly with the type of product, i.e., type of sausage, its caliber, granulation, type of casing and ripening procedure sugar employed in curing therefore plays a very important role in curing technology.

B. Acidogenic Bacteria

The acidogenic bacteria form a considerably large group of microorganisms. Of these especially lactobacilli and pediococci have been used as starter cultures in meat product technology. Both species of microbes transform meat-borne and added carbohydrates into lactic acid through homofermentation. Reduction processes are affected by lactic acid which in turn influence meat color. Bacterial activity enhances gel formation lending the product a firmer consistency. Aromatic compounds are formed by the bacterial metabolism.

The final step in the formation of lactate is the reduction of pyruvate by lactate dehydrogenase yielding NADH and the end product lactate. Lactate has an asymmetric carbon atom. Depending on the structure of the lactate dehydrogenase either L-(+)-lactate or D-(-)-lactate may be formed. The muscles of mammals contain mainly L-(+)-lactate

C. Molds

The quantity of air-dried, i.e. unsmoked fermented sausage in proportion to other types of sausage, may differ widely from one country to another. The surface of air-dried sausage is either covered with a layer of mold or yeast which lends the product its specific appearance and aroma.

Since the discovery of aflatoxins there has been a vivid debate on the possibility of deleterious effects to the health of consumers from mold layers on sausage. In addition, an often observed unpleasant discoloration of this type of sausage has kept customers from purchasing these products. In some parts of the world there

is, in fact, a pronounced aversion against moldy sausage. Many producers brush or scrub the surface of moldy sausage partially removing undifferentiated mold layers and subsequently powder the sausages with talcum, rice flour, wood ashes or other substances. However, during mold formation possible mycotoxins will already have penetrated into the product.

This situation requires selection of nontoxic molds. Despite the use of specified mold starter cultures there still is considerable neglect of this point. During recent years two procedures have been studied and have been employed in production. Inhibition of mold growth can be obtained by employing chemical preservatives such as potassium sorbate and pimaricin. A biological approach is the inoculation with spores of nonhazardous molds. Certainly, these molds must also have an esthetic appearance and be easy to handle from a technical point of view. Other commercially available starter cultures are strains of *penicillium caseicolum* (candidum) and *penicillium expansum*. Yeast starters from various species of *Debaryomyces* have also been marketed. Molds can be obtained in the form of liquid spore concentrates or as lyophilized powder. These products are easily applied as the spores suspend readily in water. Freshly stuffed sausages are simply dipped into a spore suspension and subsequently left to age and ripen.

Uniformity of the mold layer depends on an even distribution of spores through the process of 'dipping', on proper sanitary conditions and suitable humidity and temperature. Difficulties arise when wrong spore concentrations are used, when the dipping vats are not sufficiently sterilized and when air-conditioning contaminates the product. Companies changing over from unspecified molds to starter cultures will most likely find their first productions contaminated with unwanted strains. Thorough disinfection of all equipment is, therefore, necessary.

Choice of suitable casings is equally important for the uniformity of mold layers on sausage. Either natural or synthetic casings may be used. Cellulose casings may under certain conditions e.g. high air humidity and temperatures - be damaged through enzymatic activity of cellulases which are synthesized by most mold species.

Mold starter cultures have several advantages for sausage production. The sausages will have a dry, adhesive, uniform mold

layer with a whitish-grey appearance, an appealing odor and taste, and good shelf life. Rapid growth of the cultivated mold strains suppresses the development of undesirable molds, bacteria and yeasts: dry rims formed as a result of improper air-conditioning do not appear as readily. Furthermore, the mold protects the sausage from damage by intense light and high oxygen concentrations. Finally, molds prevent rancidity by synthesizing catalase: but most importantly the use of these certified molds is a guarantee for a non-mycotoxic product.

Starter Cultures and Antagonism

Life is characterized by constant interactions between organisms: this obviously applies to plants and animals but it certainly also applies to microbes. The functional ecological coexistence of bacteria within a limited biotope is referred to as biocoenosis. The species involved may either exert a positive effect on each other (synergism) or may suppress each other's growth and development (antagonism).

In food technology bacterial antagonism can be used as a tool for suppressing growth of undesirable bacteria. It may be based on two different 'strategies': a) the ability to use nutrients which other bacteria cannot utilize, a faster rate of growth than competitors which depends on the initial spore content, and b) elimination of competitors by excreting metabolites, notably antibiotics which inhibit growth of competing bacteria.

Lactobacilli and pediococci form lactic acid as a metabolic end product. Lactic acid acts as an 'anti-biotic' on a great variety of undesirable bacteria in foods which do not tolerate low pH environments: staphylococci belong to this group of low pH sensitive microbes. Below pH 5 growth of staphylococci is restricted and below pH 4.7 growth ceases. Thus, the most favorable conditions for fermentation are below pH 5.

In countries where fermented sausage is ripened at pH values higher than pH 5 pathogenic strains of staphylococci may develop. Non-pathogenic strains of staphylococci sold as starter cultures are therefore selected for acid resistance so that they can exert enzymatic activity at low pH values.

The low pH is not the sole factor leading to antagonism. Other acids have been studied. It was found, however, that at the same pH lactic acid acted more efficiently as an antagonist than other acids.

The enzyme catalase produced by staphylococci and micrococci may also act as an antagonist in fermented sausage production. As described earlier various strains of lactobacilli form peroxides which cause discoloration and rancidity of sausage: catalase inactivates these peroxides.

Antagonism has become a very important tool in sausage production and food hygiene. By employing selected starter cultures and keeping control over fermentation processes it has been possible to eliminate potentially harmful salmonellae, staphylococci and clostridia: starter cultures can thus be used to meet quality requirements in food sanitation.

Quality Control of Starter Cultures

Throughout the world starter culture are now employed for the curing of meat. Legislation in most countries does not consider starter cultures as foreign adjuncts since the species of bacteria used in starters are part of the natural bacterial flora found in classical curing procedures.

The production of starter cultures requires a high degree of responsibility. Producers must guarantee that the cultivated bacterial strains are free of substances which could negatively effect the health of the consumer. Cultures must also be suitable for large scale production. After all, the microorganisms and their metabolites are consumed along with the sausage and this certainly calls for careful routine screening of all products. All starter cultures must be constantly surveyed with regard to the absence of any kind of pathological microbe (salmonellae, staphylococci, and clostridia) and of any kind of technologically undesirable bacteria (streptococci, coliform organism and peroxide-forming bacteria).

The quality of starter cultures depends, apart from the absence of pathogenic organisms, on the fermentation activity which will be discussed below and on the total count of starter organisms. There is a definite correlation between the bacterial count and the fermentation activity.

The nutrient media included in Table 10.2 have shown in our laboratories to be most suitable for the individual groups of microorganisms. The media are standardized and yield reproducible results.

The effectiveness of starter cultures is based on their growth rate and on their ability to form required enzymes under the conditions

of the meat substrate. These factors are: nutrient availability, temperature, water content, a_w values, salt concentration, pH value, redox potential, antagonism and others. Proper strains are selected by screening bacteria naturally found in meat, sausage, and curing brines. For mixed cultures it is important to select strains which 'co-operate' and do not inhibit each other's activities.

The curing process involves two main activities: formation of acids by metabolism of meat-borne and added sugars, and nitrate/nitrite reduction. Bacterial species may only be able to carry out one or the other reaction, or both: there are acidogenic bacteria, nitrate and nitrite reducing bacteria, but also some genera which to varying degrees are able to serve both Purposes. Certainly, many other fermentative reactions occur depending on the kind of substrate and environmental conditions. In cured meat production, however, the main two parameters are acid formation and nitrate/nitrite reduction.

Table 10.2 : Starter Culture Microorganisms and Nutrient Media

<i>Microorganism</i>	<i>Nutrient Media</i>
Staphylococci	Chapman agar
Micrococci	Staphylococcus agar No. 110 Staphylococcus medium No. 110 Staphylococcus medium No. 110
Lactobacilli	Rogosa agar Rogosa-SL agar
Pediococci	MRS agar MRS agar
Yeasts	Malt extract agar Malt extract agar Malt extract agar
Streptomycetes	Caso agar Trypticase soy agar Actinomycete isolation agar Streptomycete medium Oatmeal agar
Molds	Malt extract agar Malt extract agar Malt extract agar Potato glucose agar Potato dextrose agar Potato dextrose agar Potato dextrose agar

Acid formation by lactobacilli and pedicocci starter cultures is a decisive step in meat curing but is equally important in many other branches of food production. Therefore, it has been necessary to determine the acid forming potential of the bacteria in question.

The measurement of enzyme activity is an important tool to estimate the effectiveness of starter cultures. This permits the use of starter cultures not only on the basis of cell quantity, but also on the basis of their metabolic activity.

11

Vinegar

In India "vinegar" is defined as a product which in 100 g contains a minimum of 5 g and a maximum of 15.5 g of water-free acetic acid, and which has produced

1. by acetous fermentation of liquids containing alcohol, also including dilution with water (fermentation vinegar),
2. by dilution of acetic acid with water (vinegar from acetic acid),
3. or by blending fermentation vinegar with acetic acid or with vinegar made from acetic acid.

Treatment of Raw Vinegar

It contains less than 0.5% by vol. of alcohol, and, depending on the raw material, up to 20 g acetic acid per 100 mL. Raw vinegar is more or less turbid as it contains acetic acid bacteria and material. Prior to consumption, it therefore needs further treatment.

Storage and Quality

In the course of acetous fermentation, the pH value of the fermenting mash is reduced. Vinegars obtained from natural raw material therefore show a strong lability with regard to the solubility of previously dissolved substances. This lability lasts the longer, the less pH changes. A freshly produced cider vinegar, for example, containing 5 g acetic acid per 100 mL, will give off for months substances which become insoluble - a process which develops considerably faster when apple concentrate is fermented to vinegar of approx. 10 g acetic acid per 100 mL. For this reason alone a storage of vinegar obtained from natural raw material over several months appears to be necessary. However, this does not apply to spirit vinegar. Vinegar for storage should preferably be undiluted, the way it is discharged from the fermenter.

By storing it, the quality of vinegar improves, and this also holds true to a limited extent for spirit vinegar. After proper storage there should be no difference in either smell or taste between vinegars obtained from submerged and from surface fermentation. An inferior quality of vinegars produced in generators suggests a slimy column with old shavings and an inferior quality of vinegar produced by submerged fermentation indicates mistakes in the process, such as poor selection of bacteria, insufficient aeration, or the addition of excessive quantities of nutrients. Vinegar must have a pure aroma which imparts the flavor of the raw material.

Fining

Though filters have been developed which, by filtration of the raw vinegar in one step, yield a clear and sterile product, it is common practice to fine vinegar in order to facilitate subsequent filtration and increase its stability. For fining, bentonite is used almost exclusively. It is suspended in cold water and mixed quickly and intimately with the vinegar. Its colloids are charged negatively and coagulate with the mostly positively charged acetic acid bacteria, proteins, and metal ions. Depending on the degree of turbidity, 500-3000 g of bentonite per 1000 L of vinegar are required. The optimal quantity must be found out in laboratory tests for each vinegar to be fined. If the quantity is the correct one, the turbid substances settle quickly, and the supernatant becomes clear and easy to filter. The turbid deposit can be mixed with more vinegar to be filtered and reused for fining by adding minor quantities of fresh bentonite. After repeating this procedure several times, the turbid sediment must be disposed of. The loss of acetic acid is in the range of a few percent.

Filtration

Traditional methods

Filters with diatomaceous earth coating are well suited to filter aged or non-aged, fined or unfined vinegar. A filter layer of approx. 1 mm thickness of diatomaceous earth or cellulose is placed on an adequate coat of acid-resistant steel, wood, or nylon. The quality of this filter coat determines the flow rate of vinegar filtered through the layer, and the brilliance of the product. During filtration, diatomaceous earth is added continuously to keep the growing filter cake as permeable as possible. As soon as the filtration pressure has

increased and the filter capacity has decreased too much, filtration must be stopped and the filter must be cleaned and prepared anew. Complete, largely automatic filters both with horizontal and with vertical filtering surfaces are available on the market.

Vats filled with special, pretreated wood may also be used for vinegar filtration. These vats are filled with liquid to be filtered. Each day, some 10% of the contents are drawn off at the bottom as clear vinegar, and are replaced by raw vinegar filled in at the top.

The filtered vinegar still requires sterile filtration before bottling. Either deep bed filters with finely porous discs or cartridges, or membrane filters of traditional design and construction are in use.

Continuous ultrafiltration in the Frings stream filter

To simplify filtration, a membrane filtration process has been developed which allows an automatic and continuous filtration of unfined vinegar produced by submerged fermentation and which yields a bacteria free product. The dynamic pressure of the liquid to be filtered which flows parallel to the membrane must be higher than the product from the static filtration pressure, the ratio of the largest to the smallest diameter of the particles to be filtered, and the friction coefficient of the particles on the membrane. If this condition is fulfilled, the membrane will remain free from deposits.

In laboratory tests, filtration could be performed for 250 days without one single interruption, and only a negligible reduction of the filtration capacity. Filtration pressure was 500 μ bar; flow velocity parallel to the membrane was 1.80 m/s.

For a long time, the transfer of these findings to industrial scale presented considerable difficulties. A successful result was obtained when filter modules were used which each consist of a bundle of 500 mm long, thin polyamide tubes of a diameter of 1.5 mm only through which the liquid to be filtered is passed. The filtrate which flows through the walls of the tubes is collected in a pipe surrounding the tube bundle and is drained. In the Frings stream filter, the liquid flows from top to bottom through two or three vertically spaced modules which are connected in series. The pressure drop in these modules is compensated by successive fall interval zones with the effect that all modules are operated under equal pressure conditions and thus yield optimal filtration results. A pump conveys the raw vinegar from a circulation tank to the

modules and back again into the tank. An automatic supply of vinegar to be filtered keeps the liquid level in the circulation tank constant. The filtered vinegar is collected, checked automatically and pumped off. Fig. 11.1 shows a Frings stream filter with two filtering circuits of two modules each.

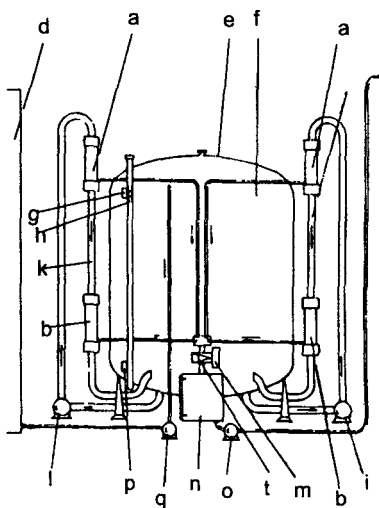


Fig. 11.1 : Frings stream filter with 2 circuits of 2 modules each and 18m² filter surface - a. and b. filter modules, c. raw vinegar pump, d. raw vinegar tank, e. circulation tank, f. level in circulation tank, g. and p. level switches, h. float, i. circulation pump, k. circulation line, l. filtrate collection, m. light barrier, n. filtrate container, o. filtrate pump.

A filter with a circulation tank of 8000 L volume and two circuits with two modules each of a total filter surface of 18 m², filters an average of some 15000 L vinegar a day. When, after 30 days of uninterrupted filtration, the concentration of bacteria in the circuit increases to a value which is approx 60 times higher than the original value, the supply of raw vinegar is interrupted, and the bacterial concentrate is thickened further. If the concentrate is additionally washed, the loss of acetic acid amounts to less than 0.2% when the concentrate is disposed of. After draining, the filter is treated with an alkaline cleaning, solution at 60 °C, followed by flushing with cold water.

Pasteurization

Vinegar made from natural raw material as opposed to spirit vinegar is frequently unstable - even after sterile filtration - at the acidities prescribed for bottling, viz, between 4 and 7 per 100 mL. This phenomenon is attributed to enzymes in the vinegar and to microorganisms which are possibly introduced into the vinegar with the water during dilution. These microorganism may later cause cloudiness in the bottles. To avoid this the vinegar is pasteurized prior to avoid this the vinegar is pasteurized prior to bottling. Pasteurization is effected either by heating to 75-80 °C for 30 to 40 seconds, or by heating to 50-55 °C for longer periods. Intermediate conditions are possible. The optimal solution depends on the type of vinegar and its constituents. At lower temperatures, only the enzymes are inactivated, and at higher temperatures, the microorganisms as well. On the other hand, higher temperatures might influence the color, smell, and taste of the vinegar, and again cause turbidities.

Sulfiting

So, is the traditional preserving agent and anti-oxidant which is allowed by law vinegar from natural raw material binds and oxidizes very quickly SO_2 added in gaseous form or as $\text{k}_2\text{S}_2\text{O}_5$. An addition immediately before bottling is therefore essential. Binding velocity depends on the raw material, especially on the free aldehyde and ketone groups, and on the pH and rH. In the bottles, free active SO_2 is mostly present in quantities of less than 10 mg/L.

Coloring and Decoloring

Spirit vinegar is of a slightly yellowish color due to the application of nutrients and due to substances liberated by the acetic acid bacteria. In a number of countries, spirit vinegar is sold completely colorless which requires a treatment with activated carbon. In other countries it is colored yellow with caramel or other colorants admitted for food. Vinegar from natural raw materials sometimes also requires coloring. Red wine, for instance, is becoming lighter during vinegar fermentation, so that occasionally, oenocyanines are used for a deeper color.

Bottling and Shelf Life

Vinegar for use in households is filled into bottles of glass, PVC or polyethylene, sealed with plastic screw caps. Air tightness is essential to guarantee preservation. Spirit vinegar does not present any difficulties, but all vinegars made from natural raw material do. Sufficient aging, clarification, sterile filtration, pasteurization, and sulfiting are the measures to choose from. Sometimes, a reduction of the content of heavy metals is also inevitable.

Use of Vinegar

In the household, vinegar is mainly used for the acidification of salads and vegetables and for seasoning meat and fish. The food industry makes use of the long-known capability of vinegar to preserve and season food at the same time.

Others found that vinegar has a specific, inhibitory effect on the growth of microorganisms, as compared to diluted acetic acid whose effect is a function of acid concentration only. The reason of this phenomenon is yet unknown.

According to studies a minimum quantity of 3.6% acetic acid is necessary for good preservation of an unpasteurized product. This value refers to the dry substance which is found after heating the product to 70 °C at pressure of 33-66 mbar. If, for instance, a vegetable contains 85% non-volatile constituents, the required minimum rate of acetic acid is $3.60 \times 0.85 = 3.06\%$.

The most important preserved vegetables prepared with vinegar are gherkins, mixed pickles, mustard, tomato ketchup, peppers, Worcester sauce, and salad dressings. Fish and meat are also preserved with vinegar.

Constituents of Vinegar

Besides acetic acid and alcohol, vinegar contains secondary constituents which play an important role for its smell, taste and preserving qualities. These constituents have their origin in the raw material, in added nutrients, and in the water used for dilution. They are also formed by acetic acid bacteria, or they are a product of fermentation or of the interaction of produced components. Their variety is surprising.

Spirit Vinegar

Workers identified, in the same spirit vinegar containing 11 g/100 mL of acetic acid, 15 µg/L of riboflavin, 83 µg/L of pantothenic acid.

Others indentified the following 1-amino acids in a spirit vinegar of 4.5 g acetic acid per 100 mL: 4.4 mg/L of isoleucine. 3.0 mg/L of leucine, 2.9 mg/L of phenylalanine. 2.5 mg/L of arginine, 2.3 m/g L of lysine, 1.1 mg/L of tyrosine, 0.58 mg/L of cystine. 0.35 mg/L of methionine, and 0.016 mg/L of tryptophan. These amino acids are secreted by the acetic acid bacteria.

In an ether-pentane extract of neutralized spirit vinegar, 27 volatile compounds could be identified, among these ethanol ethyl acetate, acetaldehyde, ethyl formate higher alcohols, but also compounds whose origin is difficult to explain, such as bromoacetaldehyde-diethylacetal.

Wine Vinegar

Scientists identified in vinegar obtained form pure wine and containing approx. 10% acetic acid, 64.5 µg/L of riboflavin, 266 µg/g of nicotinamide, and 167 µg/L of panthotenic acid. These values are essentially higher than those of spirit vinegar.

Wine vinegars contain the same spectrum of amino acids as spirit vinegar, but higher amounts. A tested wine vinegar of 4.5 g acetic acid per 100 mL contained 540 mg/L of isoleucine, 100 mg/L of phenylalanine, 88 mg/L of lysne, 88 mg/L of leucine, 85 mg/L of cystine, 50 mg/L of arginine, 19 mg/L of methionine, 13mg/L of tyrosine, and 1mg/L of tryptophan.

In an ether-pentane extract of wine vinegar, identified 42 compounds. Besides the substances which had been found in spirit vinegar, compounds derived from higher alcohols such as isopently acetate, isovalerldehyde, or β -phenethyl acetate are of particular interest. Others analyzed 20 types of wine vinegar. Most of them contained acetion and butylene glycol, but only 7 contained diacetyl.

Cider Vinegar

Workers identified 33 compounds in the ether-pentane extract of cider vinegar and analyzed at the same time the apple juice and

the cider from which the cider vinegar was made. Only few of the 33 compounds were found in the unfermented apple juice. During alcoholic fermentation with yeast, some of them were formed. During acetous fermentation, these compounds were converted into new compounds either in part or in total. They corresponded approximately to those found in wine vinegar.

Malt Vinegar

Studies identified in malt vinegar 6 esters, 6 alcohols, 2 acids, acetoin and acetaldehyde. The concentration of the volatile compounds in malt vinegar hardly varied when the concentration and the composition of the wort, the yeast strain, the culture of acetic acid bacteria, or the type of the acidification process were changed.

Others found 54 compounds in malt vinegar. In addition to the compounds identified in wine and cider vinegar, halogen compounds could be identified which obviously originated from the raw material.

Whey Vinegar

Workers found the following amino acids in whey vinegar of 4.5 g acetic acid per 100 mL: 100 mg/L of leucine, 380 mg/L of isoleucine, 110 mg/L of lysine, 50 mg/L of phenylalanine, 34 mg/L of arginine, 28 mg/L of cystine, 14 mg/L of tyrosine, 14 mg/L of methionine, and 2.3 mg/L of tryptophan.

Rice Vinegar

Scientists identified 5 phenolic acids in rice vinegar. Others found that the percentage of amino acids in rice wine decreased during acetous fermentation. Some indicate the contents of free amino acids in rice vinegar with 5 g acetic acid per 100 mL as follows: 49.7 mg/L of alanine, 45.1 mg/L of leucine, 40.3 mg/L of glutamic acid, 38.6 mg/L of aspartic acid, 35.7 mg/L of threonine, 33.3 mg/L of lysine, 29 mg/L of arginine, 29.1 mg/L of isoleucine, 25.6 mg/L of serine, 24.0 mg/L of tyrosine, 21.9 mg/L of glycine, 19.6 mg/L of proline, 17.7 mg/L of phenylalanine, 7.4 mg/L of histidine, 7.1 mg/L of methionine, 6.3 mg/L of cystine.

Analysis of Vinegar

Vinegar is analyzed for two different purposes:

- for process control by general routine methods.
- for a comprehensive knowledge of its chemical constituents by special methods.

Routine, Methods

1. Acids

Six to 20 mL of vinegar are titrated with 1N NaOH to a pH value of 8.5 with phenolphthalein as indicator. Vinegars of intensive color must be diluted with CO₂-free water before titration. 1 ml of 1 N NaOH corresponds to 0.06 g acetic acid. In such general titrations, minor quantities of other acids normally present, such as tartaric, citric, lactic, and malic acids are calculated as acetic acid.

2. Alcohol

Distillation with subsequent alcoholometric or refractometric determination of the distilled alcohol in the usual method: approx. 75 mL of 100 mL of neutralized vinegar are distilled off and collected in a measuring flask. This alcoholic liquid is then diluted to 100 mL with distilled water and is measured by means of a pycnometer, an alcoholometer, or a refractometer. Occasionally, the boiling point of vinegar is determined by an ebulliometer. Results, however, are relatively inaccurate.

While a sufficient precision is obtained with these methods at alcohol concentrations higher than 3% very low alcohol values under 0.5% may lead to substantial distortions due to the presence of measurable quantities of other volatile compounds whose characteristics are similar to those of alcohol. More precise and more specific methods may therefore and more specific methods may therefore be necessary, as for example the enzymatic alcohol analysis.

3. Sulfurous acid

Rapid analytical methods are common which are suited both for the raw material and for the finished vinegar. The sulfurous acid is titrated with 1/64 N iodine solution either for total sulfurous acid or for free sulfurous acid, using starch as indicator. More accurate results are obtained by steam distillation.

4. Sugars

Determination of sugars metabolizable by acetic acid bacteria is important for the assessment of raw material quality. For acetous fermentation the presence of sugar is indispensable, its concentration depending on the process. One method of determination is the reduction of Fehling's solution with a titration of the cuprous oxide by 0.1 N potassium permanganate.

Especially glucose is important as this sugar is the main carbon source for *Acetobacter*.

5. Ester (acetic acid ethyl ester)

Determination is occasionally performed for a quality grading of alcohols to be fermented, or for denatured alcohol which had been exposed to temperatures of 30 °C and over for months and had thus undergone esterification. In principle, the method of determination is a saponification. The ester is calculated from the difference between the acidity before and after saponification.

6. Alcoholic constituents

Occasionally, an analysis of the alcoholic constituents is required in order to decide whether distilled alcohol can be fermented or not. Distillers most frequently use gas chromatography for the determination of the following constituents: methanol, acetaldehyde, pentanol, butyric acid, residual ash, and fusel oils. The limit values of these compounds decide the quality of the alcohol.

7. Analysis of process water

A routine analysis of the process water used for the production of spirit vinegar is an important factor. Steadily growing pollution also affects natural water sources, and the result is an increasing need for chemical water treatment. Microbiological physical, and chemical total analyses of the water are decisive for its basic suitability. If such suitability is guaranteed, a routine determination of free chlorine-frequently by the quick method with the identification reagent orthotolidine - and of hardness are sufficient in most cases.

B. Special Methods

Special methods comprise a general organoleptic and biological test as well as analysis of all essential constituents of vinegar. Both

the spectrum and the concentration of the individual chemical components of vinegars may differ more or less. In principle, a distinction must be made between spirit vinegar and vinegars rich in extract.

1. Organoleptic-biological examination

These include an assessment of the smell and taste, the determination of deposits (post-precipitations as a consequence of chemical-physical reactions especially in connection with the aging of vinegars rich in extract) and the identification of vinegar eels or even mite. A test for sterility, i.e. the complete absence of bacteria of all kinds, and a microbiological identification of individual groups of bacteria plus a total bacterial count are sometimes necessary. For bacterial counting, be it to prove the existence of acetic acid bacteria, be it to identify other types of bacteria, the selection of suitable, normally solid culture media is of great importance. Special combinations of culture media with low pH are preferably used. In the course of the general progress in process technology, however, some of these examinations - excluding bacterial counting- have become less important, since a perfect product which is unobjectionable with regard to smell, tastes, eels, and mites, is today taken for granted.

2. Constituents, added substances, contamination

Not all of substances indicated below are determined with frequency. In most case, the analysis is restricted to total acid, sulfurous acid, alcohol, mineral acids, heavy metals, and preserving agents. In wine vinegar and other vinegars rich in extract, sugar-free extract, tartaric acid, sorbite, methanol (to identify the use of marc), glycerol, and actylmethylcarbinol are analyzed in addition. Further determinations are: citric acid in lemon vinegar, malic acid in eider vinegar, and phosphate in malt vinegar. In raisin vinegar, banana vinegar or other special fruit vinegars, the typical aromatic substances are frequently identified by gas chromatography.

The following analyses are usually performed.

a. Non-volatile acids

According to the analytical methods of the AOAC 10 mL of vinegar are evaporated to dryness and absorbed by distilled water. This process is repeated at least five times. subsequently, 200 mL of

freshly boiled, cooled-down water are added, and the liquid is titrated with 0.1 N. KOH. Calculation is made for acetic acid.

b) Total acid

see sect. VI.A.I

c) Volatile acids

The content of volatile acids is calculated from the difference between total acid and non-volatile acids. A direct determination of volatile acids is also possible by steam distillation: 10 mL of vinegar are diluted with 20 mL of water and are distilled in the vapor stream until 400 mL have been obtained. The distillate is titrated with 0.5 N NaOH. The non-volatile acids can be titrated with 0.1 NaOH in the distillation residue.

d) Alcohol

In the enzymatic method, the alcohol is oxidized to acetaldehyde by means of a specific alcohol dehydrogenase (BERG-MEYER, 1962), and the reduction of equimolecular quantities of formed NADPH₂ is measured at 340 nm. To determine minor quantities of alcohol, recommended a chromometric analysis in which is oxidized by potassium dichromate. However, this method is not very specific.

e) Weight ratio (density)

The weight ratio is calculated by dividing the mass of the liquid to be analyzed (vinegar) by the mass of the same volume of a reference liquid (water), weighing a pycnometer at 20 °C in an air-filled room.

f) Free mineral acids

The pH of 5% acetic acid is 2.45, of 7% acetic acid it is 2.40 and of 10% acetic acid it is 2.35. The addition of 0.25% hydrochloric acid reduces the pH of a 4% acetic acid from 2.5 to 1.6 the addition of 0.25% nitric acid to 1.8, the addition of 0.25% phosphoric acid to 1.95. Due to the presence of buffer substances, the pH of vinegar is always slightly higher, normally about 2.6-2.9 for vinegar of 5% acidity. Eventually added free mineral acid is identified by using adequate pH indicator such as methyl violet, thymosulphophthalein, or by measurements with a pH electrode.

g) Extract

A quantity of at least 50 mL vinegar is concentrated by evaporation in a platinum dish on the water bath until a syrupy

consistency is obtained. The residue is then dissolved in 50 mL water, and the solution is thickened again. This procedure is repeated at least twice. The residue is dried for 2.5 hours at 110 °C and is weighed after cooling it down in the desiccator.

h) Sugar-free extract

Frequently, the indirect calculation method is used, in which only a few chemical parameters: alcohol, extract, sugar and total acidity have to be determined.

i) Glucose

A selective and quantitative method is the enzymatic determination of glucose, the carbohydrate which for the subsequent acetous fermentation is of decisive importance as energy supplier. By a specific glucose dehydrogenase, oxidation to phosphogluconic acid takes place, and the equimolecular quantity of formed NADPH₂ is measured at 340 nm.

j) Ash, ash alkalinity, P₂O₃

The ash residue is determined after evaporation and annealing at approx. 525 °C. If necessary, determinations of the alkalinity of the ash and its P₂O₃ content are performed.

k) Glycerol

A qualitative identification is frequently made by paper chromatography while the quantitative determination can be carried out according to the periodic acid method. The US analytical methods of the AOAC also include a potassium dichromate method

l) Preserving agents

Specific, semi-quantitative fermentation tests with *Saccharomyces cerevisiae* are used for biological examination in order to detect substances which may inhibit acetic acid bacteria during fermentation. Frequently a quantitative analysis of sulfurous acid, which is still the most important preserving agent, is added. To determine total sulfurous acid, suggested a modification of the well-known alkalimetric method: the SO₂ which is liberated by steam distillation, is titrated with an alkaline solution, using a mixed indicator, DIE.

m) Formaldehyde

This compound is occasionally identified in diluted acetic acid. Its determination is performed with chromotropic acid after distillation.

n) Formic acid

Few described a method to identify formic acid by determining the products after oxidation with bromine.

o) Mehanol

The sample to be analyzed is neutralized and distilled off. By oxidation with potassium permanganate and phosphoric acid, formaldehyde is formed which can be determined quantitatively.

p) Acetone

The color complex which is formed by the reaction of the distillate with sodium cyano-nitrosyl-ferrate can be analyzed photometrically.

q) Pyridine

Workers indicate a method for quantiative determination.

r) Protein

A quantitative determination of the protein nitrogen is often performed according to the Kjeldahl methods.

s) Dextrin

The analytical methods of the AOAC indicate a qualitative determination of dextrin.

t) Acrid substances

After concentration on the water bath. the sample is extracted with ether. and the residue is organooptically tested. Occasionally, sulfuric acid, black and red pepper, mustard oil and piperine could be found.

u) Heavy metals

A quantitative determination of the most important heavy metals such as murcury, copper, silver, and cadmium is often performed with the atomic absorption spectrometer.

v) Sulfate, phosphate

A suitable method is described in the analytical methods of the AOAC

w) Caramel

Determination according to the analytical methods of the AOAC

x) Saccharin

A determination is possible

y) Butyric acid

Best determined by the enzymatic method

z) Propionic acid

Determination according to method

Vinegar in Food Law

A legal assessment of vinegar is of great importance in order to define, within the framework of national and international legislation, whether a product is synthetic acid or biologically obtained vinegar, and whether it is true wine or fruit vinegar, or a blend.

Differentiation Vinegar - Acetic Acid

The problem can be approached from two angles:

- a) The specific impurities in the acetic acid which go back to its production process (e.g. mercury) may serve as a criterion for differentiation.

However, as acetic acid is produced with a high purity these days, a secure identification has become more difficult.

- b) A characterization of the specific compounds in fermentation vinegars is therefore growing in significance. A great variety of qualitative reactions and quantitative determinations are described in the literature.

1. Potassium

Possibility of analysis

2. Potassium permanganate and iodine number

Possibilities of determination

3. Formol value

This analysis is suited for a differentiation of acetic acid versus spirit vinegar on the one hand, and wine and fruit vinegars versus spirit vinegar on the other hand.

4. ^{14}C Content

A critical presentation of this method can be found. Acetic acid normally has no appreciable ^{14}C activity because of the gradual decay of the raw material radiation (Mostly petroleum) during

millions of years. Contrary to this vinegar which ultimately has its origin in plants has a relatively high ^{14}C radioactivity as the plants, via their metabolism, are in balance with the CO_2 of the atmosphere and with its natural ^{14}C content.

However, acetic acids are found occasionally which also show a ^{14}C activity comparable to that of vinegar. A possible reason is that such acetic acid was produced through carbonization of wood. Wood is a recent material which shows a ^{14}C activity comparable to that of CO_2 in the atmosphere.

5. $^{13}\text{C}/^{12}\text{C}$ Isotopic Ratio

Based on the insight that the specific ^{14}C activity determination is a necessary, but not sufficient criterion for the differentiation of vinegars. Vinegar blends having a content of at least 15 to 20% of acetic acid can be identified as such.

The carbon of vinegar originates from plants which use atmospheric CO_2 practically all vinegar is ultimately made of the so-called C3- plants: plants in which the first measurable product of photosynthesis is the phosphoglyceric acid which is then used for the formation of carbohydrates. The CO_2 in the atmosphere has a lower ^{13}C content than the PDB-standard to which the ^{13}C content of petroleum or coal - raw material for acetic acid - is comparable. During photosynthesis, a further decrease in ^{13}C occurs, as the carboxylizing enzymes prefer $^{12}\text{CO}_2$ to $^{13}\text{CO}_2$. Therefore, acetic acid must have a higher ^{13}C content than vinegar. The average ^{13}C value of a number of samples of acetic acid produced from either petroleum or coal was 28%, while vinegars showed ^{13}C values between 21 and 22% with an accuracy of + 1%.

The authors recommend this work-intensive method together with the ^{14}C determination as an exact way to identify acetic acid and blends of vinegar with acetic acid.

6. Specific ^3H radioactivity

Studies have been made to use differing specific ^3H radioactivity values as a criterion of distinction between acetic acid and vinegar.

The background of this difference in specific ^3H radioactivity must be seen in the fact vinegar originates from plants which, via their metabolism, are in balance with the natural ^3H content of the

atmosphere. The natural isotopic distribution of hydrogen is worldwide 10^{-18} to 10^{-16} atoms of ^3H to 1 atom of ^1H . The value 10^{-18} corresponds to one tritium unit (1 T.U). For Central Europe, natural ^3H radioactivities between 100 and 200 T.U. are indicated.

Petroleum, on the other hand, consists of several million years old plant residues in which the natural ^3H radioactivity has decayed nearly completely. The acetic acid produced this way must therefore have a very low ^3H radioactivity.

7. Amino acids

For different amino acid contents earlier section.

8. Chromatography

Besides paper chromatography to identify carbohydrates, amino acids, but also organic acids, gas chromatography has lately gained significance. KAHN et al. (1966. 1972), for example performed comparative studies. They proved that there are clear characteristic differences in quality and quantity, especially with regard to esters, organic acids, alcohol, and other specific accompanying substances in different types of vinegar analyzed by spectrophotometry and by gas chromatography a number of vinegars to which differing quantities of acetic acid had been added. The author underlines the different contents of amino acids, polyphenols, and volatile acids. However, the great variety of secondary products of metabolism makes it difficult to verify additions of acetic acid which are lower than 20%.

9. Aldehydes, nitrogen, citric acid

Acetaldehyde which is always present in vinegar, may serve as a means to distinguish vinegar from acetic acid which normally does not contain any aldehyde.

Since acetic acid does not contain citric acid, while vinegars always do, a simple color reaction test is a relatively easy way of pre-analysis for differentiation.

B. Differentiation Wine Vinegar or Other Vinegars Rich in Extract-Spirit Vinegar

A safe differentiation between true wine vinegars and their blends with spirit vinegar, or generally between vinegars rich in extract and their blends, is of particular importance. Generally, a

good method is the identification of the specific fruit acids, such as tartaric acid in wine or malic acid in cider vinegar. A formol titration to identify amino acids may also be applied. On the other hand, fruit-specific acids and also amino acids can easily be added. Full analyses are often the best way to decide whether only pure wine has been used, or spirit vinegar has been added. However, and this will generally apply to the methods of differentiation indicated below, the limits of confidence for an estimate of the quantity of spirit vinegar added to wine vinegar are rather narrow.

The following analyses are in use:

1. Ratio: acidity/dry residue

Workers indicated a ratio between acidity and dry residue of always under 8 for wine vinegar, of 8 to 100 for spirit vinegar, and of higher figures for acetic acid.

2. UV- absorption

By measurements in the UV range between 225 and 300 nm, characteristic absorptions of wine and of spirit vinegars can be verified. plant-phenols are mentioned as absorbing substances.

3. Chromatography

As for the differentiation between vinegar and acetic acid, paper, acid and gas chromatography can be applied for the identification of specific wine and fruit vinegar constituents. For example, pure wine vinegars and their blends can be classified into quality groups based on the presence of a number of organic acids, primary and secondary alcohols, extract, and other values. The difficulty to reach exact statements, lies again in the fact that there are significant differences for some substances while there are none for others, so that only an exact interpretation of the full analysis allows any safe conclusions. It may therefore be assumed that blends with less than 20% spirit vinegar cannot be clearly identified.

4. Potassium

Photometric determination of potassium allows a quick classification into pure wine vinegar, spirit vinegar, and acetic acid, if the average quantities of potassium, determined as dipotassium

oxide, are considered as a basis: 4-50 mg/L for wine vinegar, 0. 1-2.5 mg/L for spirit vinegar, and under 0. 25 mg/L for acetic acid.

5. Specific alcohol indicators

An interesting outlook is the addition of an indicator - whose identity is kept secret by the authorities to the alcohol used for vinegar fermentation which can be determined exactly and selectively and thus allows an incontestable determination of the percentage of spirit vinegar blended with wine vinegar.

C. Adulteration of Wines

The problem is how to prove the use of low-quality grape pomace wine, pome wine, spoilt vinegar wine, or even synthetic wine. The addition of grape pomace wines can be proven by the identification of total phenols and catechin, as these compounds are present in appreciable quantities only if the grapes have been pressed excessively to reach a high extract .

The addition of spoiled wines can be proven by the identification of phloroglucine or phloroglucine carbonic acid. Pome wine in grape wine in grape wines can be verified by the identification of sorbitol which does not occur in grapes.

Microbial Flavors and Fragrances

The production of specific flavor and aroma chemicals by microorganisms has been recognized. Subsequently, numerous other components of flavors and aromas were shown to be the results of microbial growth.

Many microbially produced compounds may be chemically synthesized. Many other non-microbial individual components of flavors and fragrances may also be chemically synthesized. This has enabled the flavorist and the aroma chemist to reconstitute products, imparting desired taste and odor nuances. Most flavors and aromas, however, are complex mixtures of major and minor components reacting synergistically whose total effect is hard to duplicate. In addition, today's consumer prefers 'natural' products, and the labeling of a product as natural rather than as artificial is a strong selling point. As a result, companies have begun to examine the use of microorganisms for naturally synthesizing both individual chemicals and complex flavor and fragrance mixtures.

The industrial success of a microbial product is dependent either upon a high yield of that product in a reasonable period of time from a cheap, available substrate or upon the manufacture of a unique product which is in demand but difficult or impossible to obtain by other methods. Complementing this is the economical recovery of the product in usable form. Chemicals produced microbially have, in many instances, this desired uniqueness.

Flavor and Fragrance Chemicals

Due to the close relationship between flavors and aromas, *e.g.* citrus notes find application in both flavors and fragrances.

Methyl Ketones

Of all the flavors in demand, there is no doubt that chemicals that contribute to cheese flavors are most important. Numerous investigators have attributed the odor and taste of mold ripened cheese to the presence of methyl ketones, particularly methyl *n*-pentyl ketone (2-heptanone) as well as other short chain ketones such as 2-pentanone and 2-nonanone. Low concentrations of methyl ketones may also contribute fruity-spicy notes to fragrances.

The formation of methyl ketones from fatty acids was first attributed to *Penicillium roqueforti* mold spores and not to the mycelium. It was subsequently shown by that the *P. roqueforti* mycelium was capable of converting fatty acids with less than 14 carbon atoms, $\text{RCH}_2\text{CH}_2\text{CO}_2\text{H}$ (2; $\text{R}=\text{C}_3\text{H}_7$, C_5H_{11} , etc.) to methyl ketones. In every case, the acids were oxidized to methyl ketones with one less carbon atom than the original acid. It is also possible to convert vegetable oil and triglycerides to methyl ketones.

The formation of methyl ketones from fatty acids proceeds *via* interrupted β -oxidation. The reaction proceeds according to the scheme shown in Figure 12.1.

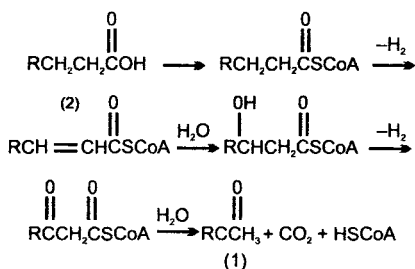


Fig. 12.1 : The formation of methyl ketones (1) from fatty acids (2; $\text{R} = \text{C}_3\text{H}_7$, C_5H_{11} , etc.); CoA = coenzyme A

Fortunately, the uniqueness of a naturally produced methyl ketone for use in fortifying cheese and other flavors may offset the manufacturing expense. Among the production difficulties encountered are the inherent toxicity of the methyl ketones towards fungi and the volatility of these chemicals. Normal fermentation techniques must be supplemented with enzymatic conversions. Since aeration during the fermentation process removes the volatile products, provisions must be made to condense vapors before they escape into the atmosphere. Residual methyl ketones may then be

easily recovered *via* solvent extraction or steam distillation. A typical process for producing 2-heptanone might proceed as shown in Figure 12.2.

Diacetyl (Biacetyl; 2, 3-Butadione)

Diacetyl, $\text{CH}_3\text{COCOCH}_3$ (3), is a naturally occurring chemical characterized by a powerful and diffusive odor resembling butter when dilute. It is extensively used in imitation butter and other dairy flavors and in numerous flavors where butter notes are desirable. Diacetyl also finds limited use in perfumes, primarily in reconstituting essential oils. Closely related to diacetyl is acetoin (3-hydroxy-2-butanone; $\text{CH}_3\text{COCH}(\text{OH})\text{CH}_3$, 4). Acetoin is frequently found with diacetyl but probably contributes little or no flavor by itself. For many years it had been assumed that diacetyl was produced from acetoin by microbiological oxidation. Numerous recent studies have shown that this is not the case. Scientists summarized the following: (1) diacetyl is not produced from acetoin by either bacteria or yeast; (2) the mechanisms by which bacteria and yeast produce diacetyl are the same, but differ from the mechanisms used to produce acetoin; and (3) diacetyl cannot be detected in several microorganisms which produce acetoin.

It has been accepted that citric acid is a precursor for both diacetyl and acetoin. In addition to the precursor citrate, the production of diacetyl is enhanced by a pH below 5.5, low temperature and aeration. A pH below 5.5 favors citric acid permease activity and restricts diacetyl reductase activity. Aeration promotes both the formation and accumulation of diacetyl by increasing the oxidation-reduction potential of the culture. This results in enzymatic stimulation and the spontaneous oxidative decarboxylation of α -acetolactic acid to diacetyl.

The various theoretical pathways to diacetyl synthesis have been summarized. The routes are shown in Figure 12.3.

Workers demonstrated that diacetyl could be obtained from aerated baker's and brewer's yeasts acting on a saccharose (sucrose) substrate. It was not indicated whether the yeast first hydrolyzed

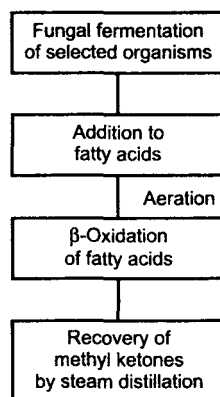


Fig. 12.2 : Flow chart for the production of methyl ketones

saccharose *via* the enzyme invertase to glucose and fructose. Today, the organisms of commercial significance which produce diacetyl are *Streptococcus lactis* sp. *diacetylactis* and several *Leuconostoc* species. The use of a humectant, such as glycerol or sucrose, which lowers the A_w value (water activity) of the medium results in greater diacetyl production.

Sucrose was converted in 70% yield to a mixture of diacetyl and acetoin. Further oxidation of acetoin by iron (III) sulfate and iron (II) chloride gave an overall recovery of 60% diacetyl on the basis of sugar utilized.

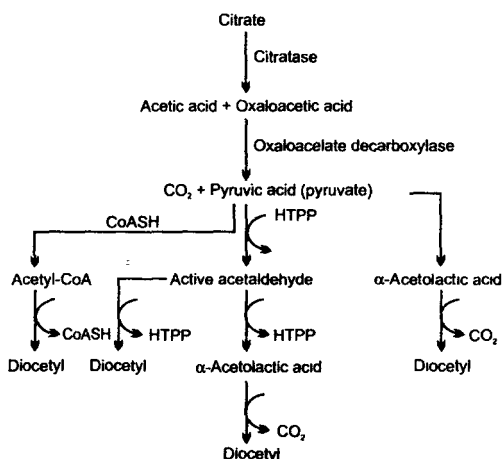


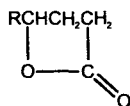
Fig. 12.3 : Pathways in diacetyl synthesis : HTPP - α -hydroxyethylthiamine pyrophosphate (active acetaldehyde)

Natural diacetyl may also be obtained by distillation of starter distillate, a by-product from the manufacture of starter cultures. Diacetyl is sold commercially in the United States as a natural product imported from France. Although exact details of the manufacturing procedure are not available, it must be assumed that this imported diacetyl is bacterial in origin.

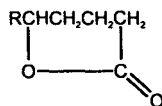
Lactones

Lactones are internal (cyclic) esters of primarily γ - and δ -hydroxy acids (5 and 6). Lactones are ubiquitous in food, contributing taste and flavor nuances. Numerous odor and taste characteristics have been attributed to lactones. Among these are oily-peachy, creamy,

fruity, nut-like, coconut, honey, and so on. Maga summarized the types of lactones identified in fruits and vegetables. Many of these have been chemically synthesized, finding use in a variety of artificial flavors. The use, however, of microbially produced natural lactones in flavors would find wide acceptance among consumers.



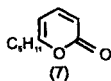
(5)



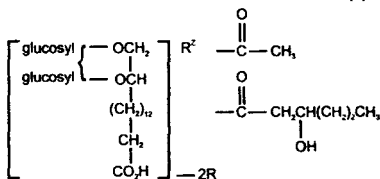
(6)

We discussed the production of lactones by yeast of the genus *Pityrosporum* and applied for a patent for the 'Production of gamma-lactones rich flavor additives by *Pityrosporum* species cultured on lipid rich substrates'. The substrates include triolein, sebum, lecithin, oleic acid and Tween 80. Among the lactones claimed are the γ -hexa-, γ -hepta-, γ -nona-, γ -deca-, γ -undeca- and γ -dodeca-lactones. Some also noted the volatile components of cultures of *Ceratocystis moniliformis* as having 'fruity-banana', 'peach-pear', and 'citrus' aromas. Identification of these by gas chromatographic head space analysis revealed a number of compounds including γ - and δ -decalactone. The above lactones are known for their peach-like aroma.

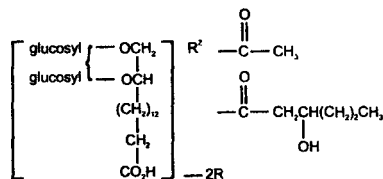
A coconut aroma is highly desired by flavorists. γ -Octalactone and γ -nonalactone possess this aroma. Another lactone having a coconut odor is 6-pentyl-2-pyrone (7). This chemical was found to be the major volatile constituent of the fungus *Trichoderma viride*. It is also claimed that the spores rather than the mycelia are responsible for the aroma. A natural microbial synthesis of coconut chemicals would have considerable impact upon the market.



(7)



(8)



(9)

Workers have demonstrated that *Ustilago zaeae*, a corn smut fungus, may be used to produce ustilagic acids A (8) and B (9), potentially useful as precursors for the synthesis of lactonic macrocyclic musks. Macrocyclic musks are extensively used in the perfume industry.

The production of ustilagic acid has been scaled up in 200 gal fermenters. Yields of 22 g l⁻¹ of fermentation broth have been obtained. Ustilagic acid, a mixture of monoacidic D-glucolipids, may be converted into its respective ustilic acids. Ustilic acid A (15D, 16-dihydroxyhexadecanoic acid; 10; Figure 12.4) can be converted to 15-hydroxypentadecanoic acid (11) which in turn can be transformed into the macrocyclic musk 15-pentadecanolactone (12), known commercially as Exaltolide®. Ustilic acid A may also be converted to cyclopentadecanone (Exaltone®)

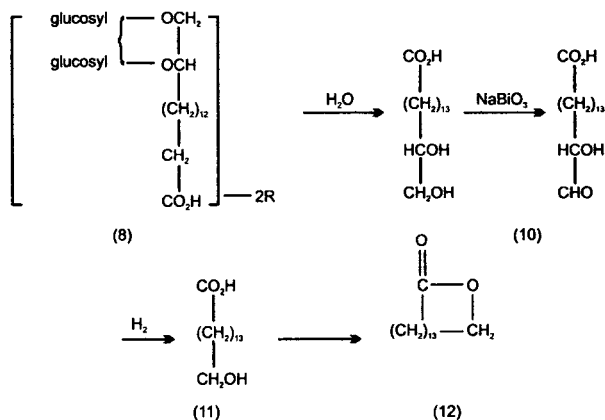


Fig. 12.4 : Production of Exaltolide® (15-pentadecanolactone; 12) from ustilagic acid A (8; R = -C(O)CH₂-C(O)CH₂CH(OH)(CH₂)₂CH₃)

In addition to the above, workers described a fermentation process for 15- and 16-hydroxypalmitic acids. These products are intermediates for the valuable macrocyclic musks 15- and 16-hexadecanolides. The procedure utilizes *Torulopsis magnoliae* to hydroxylate palmitic acid. The hydroxypalmitic acids formed are then cyclized to produce the lactones.

Butyric Acid

Butyric acid, CH₃CH₂CH₂CO₂H (13), at low concentrations is used to supply butter-like notes to flavors. It finds particular

application in natural cheese flavors. The esters of butyric acid also may contribute to the flavor of various products. Pentyl butyrate provides a strong, ethereal, fruity odor reminiscent of apricot, banana and pineapple; isobutyl butyrate supplies an ethereal, fruity, somewhat pungent odor suggestive of pear, pineapple and banana.

Although natural butyric acid as an ester may be found at a concentration of 2-4% in butter, its isolation is an expensive and difficult process. As a result, the fermentative production of natural butyric acid is a valuable alternative.

Butyric acid is primarily produced by obligate anaerobes of the genera *Clostridium*, *Butyrivibrio*, *Eubacterium* and *Fusarium*. The clostridia, particularly *C. acetylbutyricum*, have been studied in detail. Their ability to produce organic solvents such as acetone and butanol has led to commercial processes which may be modified and adapted to produce butyric acid.

The mechanism for butyric acid production has been summarized (Figure 12.5).

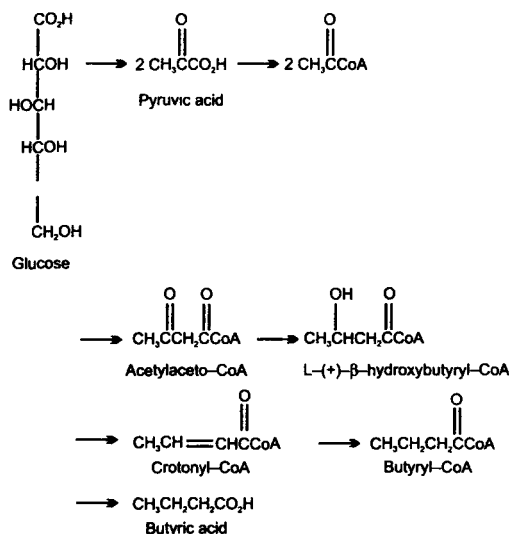


Fig. 12.5 : Mechanism of butyric acid production.

Besides proper selection of the microorganisms, it is necessary to maintain the pH above 5.0 in order to direct the fermentation away from solvent formation and towards butyric acid formation.

They showed that calcium carbonate may be used to control the pH above 5.0. When other methods of controlling pH were attempted, butyric acid yields were low, indicating that calcium carbonate plays another role, possibly serving as a point of attachment for the microorganisms. Using a simple medium consisting of cerelese (commercial dextrose), dried yeast and calcium carbonate, yields of 1% butyric acid were obtained.

Isovaleric Acid

Isovaleric acid, $(\text{CH}_3)_2\text{CHCH}_2\text{CO}_2\text{H}$ (14), is undoubtedly one of the most offensive odors encountered in the flavor/fragrance industry. Not only does it possess an acid-acrid odor commonly described as 'locker room' or 'dirty feet', but isovaleric acid has a tenacious affinity for the skin. Despite this, in extremely dilute concentrations isovaleric acid becomes agreeable and herbaceous. Furthermore, its esters find widespread use in the flavor industry. Prominent among these are ethyl isovalerate, which possesses a powerful apple-fruity odor and finds application in numerous fruit flavors as well as in candies and chewing gums; isopentyl isovalerate, which has a fruity-apple-raspberry odor and finds use in apple flavors and as a modifier in numerous fruit and nut flavors; and isobutyl isovalerate, also apple-raspberry-like and used not only in fruit flavors, but also in perfumes for lipsticks.

Isovaleric acid may be produced synthetically by the oxidation of isopentyl alcohol. Subsequent direct esterification leads to the various esters. If natural isovaleric acid is desired, this process is not suitable. Two potential methods exist for the microbial production of natural isovaleric acid. The first, the microbial oxidation of isopentyl alcohol is theoretically possible since the oxidation of terminal alcohol groups to their corresponding carboxyl groups is well known. This procedure is limited both by the toxicity and the low solubility of isopentyl alcohol. Furthermore, no microorganisms are known at present that will oxidize isopentyl alcohol. Nevertheless, the method remains an interesting challenge. The second possibility involves the conversion of leucine to isovaleric acid. Numerous investigators have demonstrated that this is possible *via* the Stickland reaction. The reaction employs amino acid utilizing anaerobes to facilitate coupled oxidation-reduction between pairs of amino acids. One amino acid is oxidatively

deaminated and decarboxylated; the other is reductively deaminated. The Stickland reaction is summarized in Figure 12.6.

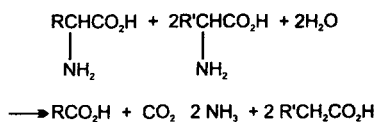


Fig. 12.6 : The Stickland reaction.

Workers using cell suspensions demonstrated that several species of the genus *Clostridium* and *Peptostreptococcus anaerobius* convert leucine to both isovaleric acid and isocaproic acid in a mechanism compatible with the Stickland reaction (Figure 12.7). In this case, leucine serves as both the proton donor and acceptor within one reaction. If however, the clostridial species does not produce isocaproic acid, leucine functions only as a proton donor, and proton acceptors must be supplied.

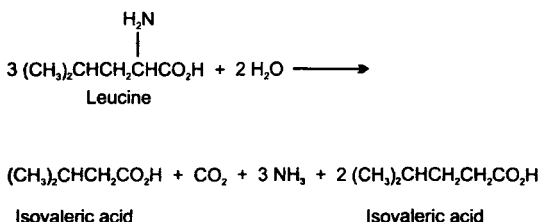


Fig. 12.7 : Microbial conversion of leucine to isovaleric and isocaproic acids.

Scientists showed that by proper selection of the medium and clostridial species, good conversions of leucine to isovaleric acid may be obtained.

Others described an interesting method of obtaining isovaleric acid. Cultures of *Pseudomonas aeruginosa* were initially grown on heptane. A small amount of chloramphenicol was then added with the substrate, 2-methylhexane, resulting in the conversion of the substrate to 30-40% isovaleric acid. It is assumed that chloramphenicol prevents the metabolism of intermediate products so that further oxidation is blocked.

Terpenes and Terpene Transformations

Terpenes are natural product whose basic structure consists of isoprene units linked head to tail (Figure 12.8)

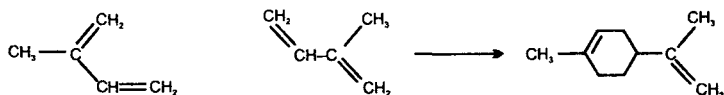


Fig. 12.8 : Production of the monoterpene (±) limonene by head to tail linkage of two isoprene units.

Terpenes are the basic components of essential oils and find widespread use in flavors and aromas. Other terpenes, including the carotenes and gibberellins, are discussed elsewhere.

The general structural formula for terpenes, is $(C_5H_8)_n$. For the monoterpenes, $n=2$, for the sesquiterpenes, $n=3$, and so on. Also found in the plant world are the closely related cyclic alcohols and ketones such as menthol ($C_{10}H_{20}O$) and menthone ($C_{10}H_{18}O$). Complete discussions of the chemistry of terpenes may be found in the books listed in Section 47.6.

Microorganisms have the unique ability not only to degrade or transform terpenes, but also to synthesize them. In addition, microorganisms may be used to racemize terpenes.

Following the initial studies involving the synthesis of various monoterpenes by the ascomycete *Ceratocystis variispora*, numerous other microorganisms were shown to synthesize terpenes. Table 12.1, compiled, lists the terpenes and some other products produced directly by fungi.

Table 12.1 : Fragrance Compounds from Microorganisms

Microorganism	Fragrance	Chemical
<i>Ascoidea hylacoeti</i>	Fruity, rose	β -Phenylethanol, furan-2 carboxylate
<i>Ceratocystis moniliformis</i>	Fruity, banana, peach, pear, rose	3-Methylbutyl acetate, δ - and γ -decalactone, rose geraniol, citronellol, nerol, linalool, α -terpineol
<i>Ceratocystis variispora</i>	Fragrant, geranium	Citronellol, citronellyl acetate, geranial, neral, geraniol, linalool, geranyl acetate
<i>Ceratocystis variispora</i>	Fragrant, fruity	6-Methyl-5 hepten-2-ol acetate, citronellol, linalool, geraniol, geranyl acetate
<i>Inocybe corydalina</i>	Fruity, jasmine	Cinnamic acid methyl ester
<i>Cluyveromyces lactis</i>	Fruity, rose	Citronellol, linalool, geraniol

contd...

Table 12.1 contd...

Microorganism	Frangrance	Chemical
<i>Mycoacia uda</i>	Fruity, grassy, almond	<i>p</i> -Methylacetophenone, <i>p</i> -tolyl-1-ethanol, <i>p</i> -tolylaldehyde
<i>Penicillium decumbens</i>	Pine, rose, apple, mushroom	Thujopsene, 3-octanone, 1-octen-3-ol, nerolidol, β -phenylethanol
<i>Phellinus</i> species	Fruity, rose, wintergreen	Methyl benzoate, methyl salicylate, β -phynylethanol, γ -Decalactone
<i>Sporobolomyces odorus</i> <i>Streptomyces odorifer</i>	Peach Earthy, camphor	trans-1, 10 Dimethyl-trans-9-decalol, 2-exohydroxy-2-methylbornane
<i>Trametes odorata</i>	Honey, rose, fruity, anise	Methyl phenylacetate, geraniol, nerol, citronellol
<i>Trichoderma viride</i>	Coconut	6-Pentyl-2-pyrone

Undoubtedly, there will be numerous additions to this list in the future. Microbiological production of terpenes, however, cannot compete economically at present with the recovery of terpenes from essential oils. Nevertheless, improvements in yield combined with a diminished supply of natural plant terpenes may make the microbiological production of terpenes an attractive possibility. Already, some have demonstrated with *C. variispora* that terpene yields approaching 2 g l^{-1} could be obtained by fermentation provided that toxic end products were removed using ion exchange resins. Further improvements would increase the practicality.

Monoterpenoids

Microbial transformations of monoterpenoid compounds are summarized in Table 12.2.

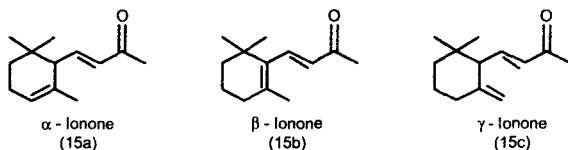
Ionones

Transformation of β -ionone (15b) has been studied. Using *Aspergillus niger*, Mikami demonstrated that β -ionone could be transformed to a complex mixture resembling an essential oil with an odor similar to that of tobacco. He suggested that these products could be used as tobacco flavoring compounds. Among the transformation products were various hydroxy- and oxo-ionone

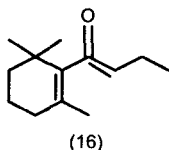
derivatives. Krasnobajew showed that *Lasiodiplodia theobromae* could also transform β -ionone into a variety of essential oil type product with a tobacco like odor. In this case, the molecule is degraded by a Baeyer-Villiger type oxidation. The main product is β -cyclogeraniol.

Table 12.2 : Microbial Transformations of Monoterpenoids

Substrate	Organism	Major product (s)
<i>Acyclic monoterpenoids</i>		
Citronellal	Yeast	(+)-Citronellol
	<i>Candida reukaufii</i>	(-) β -Citronellol
	<i>Pseudomonas aeruginosa</i>	Citronellic acid
	<i>Ps. digitatum</i>	Menthol
Citral	<i>Ps. convexa</i>	Geranic acid
Linalool	<i>Ps. pseudomallei</i>	Camphor
	<i>Ps. incognita</i>	Various cyclic compounds and acid
<i>Monocyclic monoterpenoids</i>		
Limonene	Pseudomonad	Carveol, carvone, dihydrocarvone, perillyl alcohol etc.
(+) -Limonene	<i>Penicillium digitatum</i>	Carvone
	<i>P. italicum</i>	Carvone
	<i>Cladisporium</i> sp. T-7	Limonene-1, 2-diol
	<i>Cladisporium</i> sp. T-12	(+) - α - Terpineol
(±) -Menthol (acetates)	<i>Trichoderma viride</i>	1-Menthol
	<i>Rhodotorula mucilaginosa</i>	1-Menthol
	<i>Arginomonas non-fermentans</i>	1-Menthol
(±) -Menthol (lactic acid esters)	<i>Bacillus subtilis</i>	1-Menthol
	<i>Bicyclic monoterpenoids</i>	
α - Pinene	<i>Aspergillus niger</i>	(+) -Verbenone (+) -cis-Verbenol (+) -trans-Sobrerol
α - Pinene	<i>Ps. maltophila</i>	2-(4-Methyl-3-cyclohexenylidene) propionic acid
β -Pinene	<i>A. niger</i>	Pinocarveol, pinocarvone, mystenol
1,8-Cineole (eucalyptol)	<i>Ps. flava</i>	2-Oxocineole, isomers of 2-hydroxycineole, 5,5-dimethyl-4(3'-oxobutyl)-4,5-dihydrofuran 2-(3H)-one



Workers demonstrated that α -ionone (15a) could also be transformed by *L. theobromae*, although not as readily as β -ionone. Similarly, essential oil type products with a tobacco odor were obtained. These compounds had previously been reported as flavor components of burley tobacco.



β -Damascone

The microbiological transformation of β -damascone (16) has been reported. Using fungi of the genera *Aspergillus*, *Botryosphaeria* and *Lasiodiplodia*, β -damascone was transformed to 4-hydroxy- and 2-hydroxy- β -damascone. The metabolic products were found to be useful as tobacco flavors.

Sesquiterpenoids

Sesquiterpenes are found in essential oils along with monoterpenoids. Although there are at least 2000 sesquiterpenes found in nature, reports on their microbial transformation are relatively rare. Of interest is the transformation of valencene (17) to nootkatone (18; Figure 12.9)

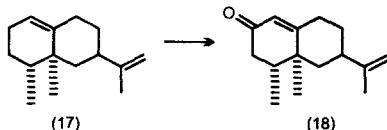


Fig. 12.9 : Conversion of valencene (17) to nootkatone (18)

Valencene, readily available from orange oil, is in itself of little commercial use. Workers showed that both a bacterium isolated from Dutch soil and a bacterium isolated from infected beer were capable of transforming valencene to nootkatone, a main flavoring

ingredient of grapefruit. The commercialization of this may be of considerable potential.

Patchouli alcohol

Patchouli alcohol or patchoulol (19) is an important ingredient of the patchouli oil used in perfumery. The odor carrier in patchouli oil is thought to be norpatchoulol (20). 10-Hydroxypatchoulol can be chemically converted to norpatchoulol. Extensive screening of microorganisms revealed that many would hydroxylate the C-10 methyl group (Figure 12.10). Numerous other hydroxylation products were also found.

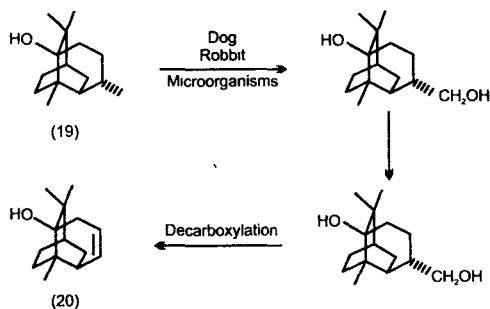


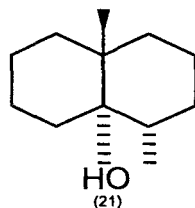
Fig. 12.10 : Conversion of patchoulol (19) to norpatchoulol (20)

Curvularia lunata was found to produce 8-hydroxypatchoulol, a precursor for patchoulion, an important trace component of patchouli oil. Although yields of valuable transformation products of patchoulol are low, optimization of the fermentation and further strain selection might yield practical amounts of 8-10 hydroxypatchoulol.

Geosmin

Geosmin (*trans*-1, 10-dimethyl-*trans*-9-decalol; 21), first isolated is an earthy smelling chemical produced by blue-green algae, myxomycetes, actinomycetes and other microorganisms. Geosmin has frequently been found contaminating water supplies. Not only does this result in unpalatable water, but also fish and animals drinking the water become unacceptable for consumption. Geosmin has two unique properties. Its odor is detectable in water at a concentration of $0.2 \mu\text{g l}^{-1}$ (0.2 p.p.b.), and it has the ability to 'fatigue' the nose rapidly, making organoleptic evaluation difficult.

The earthy notes of geosmin are useful in imparting or modifying amber notes in perfume. At a concentration of 1-100 p.p.m., geosmin is described as being useful for the reconstitution of natural essential oils.



Scientists produced geosmin in fermenters utilizing *Streptomyces griseus* LP-16 in a soybean meal-peptone-salt-glucose medium. Yields were 6 mg l⁻¹ after three days fermentation. Geosmin was recovered by extracting a steam distillate with methylene chloride and obtaining the geosmin directly by preparative gas chromatography. Since the chemical synthesis of geosmin is complicated, its production by fermentation might be a practical commercial process.

Dairy Flavors

Dairy flavors, primarily cheese, are widely used in the food industry. Cheese flavors find application in snacks, sauces, baked goods and numerous other products. To a lesser extent, yogurt and buttermilk flavors are also useful. Research has been directed towards shortening the aging time and enhancing the flavor potential of natural cheese. In the United States, cheddar cheese and its milder varieties (process, American cheese) dominate the market.

Some has discussed the biogenesis of cheese flavors. The flavor results from the action of microorganisms and enzymes on the proteins, fats and carbohydrates of the milk and curd. The numerous breakdown products such as short chain fatty acids, acetic and lactic acids, alcohols, aldehydes, ketones, esters, ammonia, amines, sulfides and mercaptans all contribute to the flavor. The characteristic flavor of various cheese is determined by the concentration and ratio of these compounds.

Raising the storage temperature speeds up flavor formation, but off flavors are promoted and microbial spoilage occurs. If the flavor of blue cheese is required, commercial *Aspergillus* enzyme preparations may be used to accelerate fatty acid and δ -lactone formation. Incubating high-moisture curd slurries with *Penicillium* at 30°C results in a flavor suitable for dips or other foods. More subtle flavors like cheddar require a balanced selection of enzymes and many investigators have attempted to do this by adding

microbial proteinase. If this process is not carefully controlled, however, bitter-tasting peptides may mask the desired cheddar flavor. The neutral proteinase of *Bacillus subtilis* gives good organoleptic results, achieving an overall cheddar flavor in approximately half the time as natural ripening. Recently, commercial preparations of β -galactosidase (lactase) have been used to convert lactose to glucose and galactose in milk and whey.

When applied to cheesemaking, glucose supposedly stimulates the growth and production of starter bacteria, resulting in a 50 to 70% reduction in ripening time. On the other hand, it has been suggested that a contaminating, heat-resistant proteinase may be responsible for the accelerated cheese ripening, since glucose itself stimulates starter cultures but does not accelerate ripening.

Non-toxic lactic acid-producing organisms of the genus *Streptococcus* and non-toxic *Acetobacter* were grown in a reconstituted milk medium with mixing and aeration. After 24 hours fermentation, the mixture was pasteurized and then either used directly or spray dried and incorporated into food products. Further modification of this process utilized non-toxic organisms of the genera *Bacillus* and *Streptococcus* to ferment milk. Naturally produced cheddar cheese was added and, after an additional day of fermentation, the mixture was pasteurized and spray dried. The resulting powder had a complete cheddar cheese taste and desirable texture. The mixture was fermented with a lactic starter culture, and then buttermilk, acid whey and sweet whey were added. Following pasteurization, the mixture was spray dried to yield a product with a strong, cheese-like flavor.

Workers claimed that *Penicillium roqueforti* could be grown rapidly in milk and that the resulting products without further aging could be used directly for flavoring foodstuffs with a blue cheese type flavor. Others modified this process by substituting sodium caseinate and butterfat for milk, avoiding some of the flavor defects in the milk process.

Bread Flavors

The flavor of white bread is dependent upon four factors. These are (1) the ingredients; (2) yeast and bacterial fermentation products; (3) mechanical and/or biochemical degradation; and (4) thermal reaction products. There are at least 100 volatile flavor chemicals in

bread many of which are yeast or bacterial fermentation products. These chemicals, when heated, give bread its final flavor and its appealing character.

Thrusts at enhancing bread flavor have taken two directions. In the first, attempts have been made to develop a preferment flavor. Some described a 'stable ferment process' designed to replace the more time consuming sponge method currently in use. A mixture of yeast, water, yeast food, sugar, salt and non-fat dried milk solids was allowed to ferment for six hours. The mixture was then added directly to dough and the dough processed, omitting the sponge step. Few suggested the use of selected microorganisms; particularly *Lactobacillus bulgaricus* plus a buttermilk culture, in preferments or in the sponge to enhance bread flavor. Others grew yeast or yeast-cocci in a whey culture, claiming that a synergistic effect occurs among yeast, enterococci of serological group D, streptococci of group N, and lactobacilli with respect to bread flavor. After fermentation, the mixture was concentrated, spray dried and added to dough. The lactic acid may be added directly or developed from lactic acid-producing microorganisms. Others fermented yeast in a sugar and maltol mixture. The yeast was allowed to autolyze after fermentation. The resulting liquid 'could either be added directly to chemically leavened dough or dried and incorporated into the product.

In the second, various enzymes preparations including microbial amylases and proteases have been recommended for improving the manufacture of bread. Some described a method for treating flour to substantially reduce development time during mixing to form the dough. The main function of enzymes, however, is to improve dough characteristics rather than improve flavor.

Mushroom Flavors

Commercially-important mushrooms belong to the orders Ascomycetes and Basidiomycetes. The Ascomycetes are represented by the truffles (*Tuber* sp.) and the morels (*Morchella* sp). The Basidiomycetes are represented primarily by *Agaricus bisporus* and *A. bitorquis*, the Shiitake (*Lentinus edodes*), the Paddy straw mushroom (*Volvvariella volvacea*), *Pleurotus* sp., *Coprinus fimetarius* and *Flammulina velutipes*.

Numerous studies have confirmed that 1-octen-3-ol, $\text{H}_2\text{C}=\text{CHC}(\text{OH})\text{C}(\text{CH}_2)_4\text{CH}_3$ (22), is the main chemical responsible for the mushroom aroma, although numerous other chemicals including glutamic acid and 5'-guanylic acid modify the flavor giving each mushroom species its distinctive odor. The search for a mushroom flavor has not, however, concentrated on producing these chemicals or combinations of them, but rather on growing the mushroom mycelium in submerged culture and then utilizing the dried mycelium as a flavorant.

Almost all mushrooms are capable of growing in submerged culture. Others grew 20 different varieties in a chemically defined medium. Samples of *Agaricus campestris* mycelium were cooked and submitted to a panel of tasters. Of the other species, only *Lipiota rachodes* had a pleasant flavor. Mycelium grown on a solid medium often has a typical mushroom odor not detectable in submerged growth. It is doubtful, however, that such a process has been developed which permits the consistent production of mycelium with the true flavor nuances of fleshy mushrooms. The flavor intensity is roughly 18 times that of whole fresh mushrooms and about 12 times that of canned mushroom slices. Enhancement of flavor is also claimed. Whether the concentrate is produced by submerged fermentation or by another process is not known.

Production of Flavor and Aroma Chemicals by Plant Tissue Culture

Various natural flavor, and aroma chemicals, e.g., vanilla and peppermint, may eventually become scarce due to economic or political situations. It is obvious that plant tissue culture should be exploited for the production of these compounds. There are three ways to utilize plant tissue culture for the production of useful products: (1) direct extraction of compounds from the cells or the medium; (2) biotransformation; and (3) enzymatic synthesis. To this may be added a fourth: utilization of the entire cellular mass for further processing. By far, most studies have been involved with the direct isolation of compounds and, of these, most have been aimed at producing chemicals of medicinal value.

Table 12.3 lists aromatic flavor substances produced by plant cell cultures.

Table 12.3 : Biosynthetic Products of Plant Cell Cultures.

<i>Substance</i>	<i>Species</i>	<i>Culture type</i>
Cinnamic acid	<i>Nicotiana tabacum</i>	C
4-Hydroxy-3-methoxybenzoic acid	<i>Linum usitatissimum</i>	C
Caryophyllene	<i>Lindera strychnifolia</i>	C
2-Undercanone, 2-undecanyl acetate	<i>Ruta graveolens</i>	C
Stevioside	<i>Stevia rebaudiana</i>	C
Limonene, Linalool	<i>Perilla frutescens</i>	C
Anethol	<i>Foeniculum vulgare</i>	C
'Ess. oil'	<i>Pimpinella anisum</i>	C
Diallyl disulfide	<i>Allium cepa</i>	C
Farnesol	<i>Andrographis paniculata</i>	S
2-Phenylethylglycoside	<i>Tropeolum majus</i>	S
Glycyrrhizin	<i>Glycyrrhiza glabra</i>	S
'Apple aroma'	<i>Malus silvestris</i>	S
L-Glutamine	<i>Symphytum officinale</i>	S

*C = callus culture; S = suspension culture.

All the techniques applicable to microbial fermentations may be utilized for the production of flavor and aroma chemicals by plant tissue culture. This includes the selection of overproducing cells, the use of precursors, medium improvement and hormone application. The commercialization of such processes depends, however, on economic considerations and it is this more than anything else that will limit the application of plant tissue culture in industry.

13

Distilled Beverages

The history of the art of making distilled beverages is shrouded in antiquity. The Chinese are generally credited with being the first to produce distilled beverages from rice beer (ca. 800 B.C.). Sometime shortly after the birth of Christ, the Egyptians began studying distillation. The Arab alchemists reportedly learned the art from the Egyptians and further developed the process. The Arabs described different methods of distillation and developed an apparatus called an alambic. The alambic had a closed container in which the fermented liquid was heated and the vapors were transferred through a tube to a cooling chamber where they were condensed. Later, the heating chamber was enclosed and a long crane neck was used to deliver the vapors to a cooling coil for condensation. The word "alcohol" was coined to describe the distillate from the alambic still. The technology for some of the stills used today is based on the alambic design. A sketch of an early pot still is shown in Fig. 13.1. Direct heat is applied to the fermented mixture in the pot, and the vapors rise into the head, passing through the lyne arm through the coiled tube into the worm tub where they are condensed. The condensate is drained into a product tank.

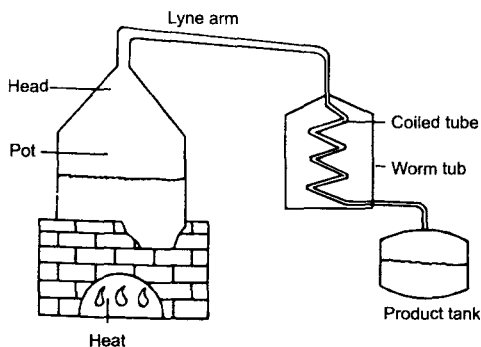


Fig. 13.1 : Schematic drawing of a pot still system.

Wine was first distilled in Germany around 1100, and the distillate was called a "wine spirit".

The Arabs were responsible for spreading the distillation art to western Europe where it was first used by the alchemists and monks, ca. 1400. The European alchemists felt that the distillate was a new element which was called water of life in Europe: usequebaugh in Ireland aqua vitae in Italy: or eau de vie in France. The Gaelic version was actually a cordial made from aniseed, cloves, nutmeg, ginger, caraway seeds, raisins, licorice and safron.

As stated earlier, the beginning stills were called alambics. They were modified and improved to become pot stills, and improved versions are in use today in Scotland for malt whiskey production and in France for brandy. The Coffey still was invented in 1830 by AENCAS COFFEY, in Dublin, Ireland. This utilized a continuous, allowing a faster and cheaper distillation at higher proofs and a better quality distillate than the pot still.

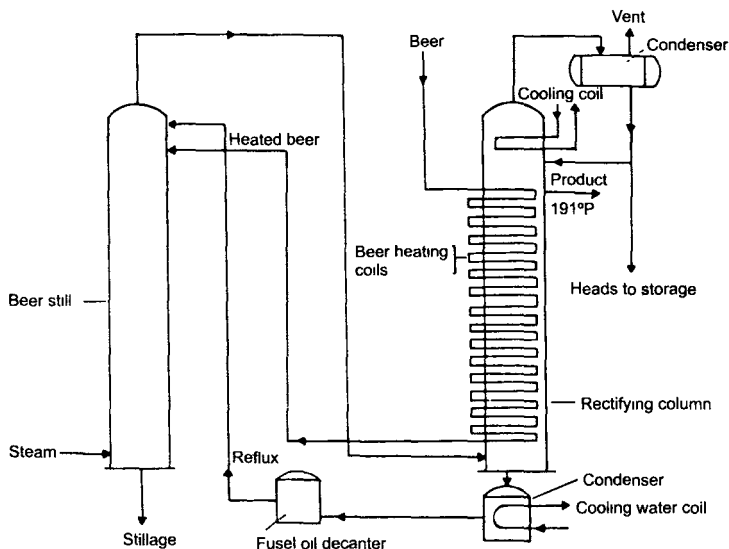


Fig. 13.2 : Diagram of a Coffey still.

The Coffey still (Fig.13.2) is composed of two columns, the rectifier and the beer still. The beer flows through the coiled pipe in the rectifying column being heated by up, coming steam, then is sprayed onto the top plate of the beer still. As the liquid falls

downward and meets the upcoming steam, the alcohol evaporates, vapor flows to the drained from the base of the beer still. The vapors condense and gradually the alcohol concentration increases as the liquid rise close to the top of the rectifier column where it is drawn as product. The lowest boiling components (heads) are drawn as vapor to be condensed and recycled.

The coffey still is in use today both in Europe and in the United States. More recent improvements have been the beer still and doubler, which resembles a pot still, and the multi-column units for producing neutral spirits for gin and vodka.

Definition of Products

Distilled beverage spirits may be classified by the type of raw material used, the process by which the spirit is produced and the country of origin. Neutral spirits are produced from any material. distilled at or above 190° proof and bottled at 80° proof or higher. In India unless spirits are made from grain, the substrate has to be specified. Proof describes the alcohol content of a distillate, which is twice the percentage of alcohol contained, e.g. a 100° proof distillate is 50% alcohol on a volume basis.

Vodka is a neutral spirit so distilled or so treated after distillation, by charcoal treatment, as to be without any distinctive character, aroma, taste or color. In the United States, if it is not made from grain, or if any flavoring is added, it must be so stated.

Grain spirits are neutral spirits distilled from a fermented grain mash and stored in used oak barrels and bottled at not less than 80° proof.

Whiskey is a general term to describe a product distilled from a grain mash at less than 190° proof in such a manner that the distillate possesses the taste, aroma, and characteristics generally attributed to whiskey, and stored in oak barrels, except corn whiskey, which is stored in used barrels.

Bourbon, rye, wheat, malt or rye malt whiskeys are distilled at not over 160° proof from a fermented mash of not less than 51% of corn (Bourbon), rye, wheat, malt or rye malt, respectively, stored at not more than 125° proof in charred new oak containers for four years, and bottled at not less than 80° proof. If less than four years it must be stated.

Corn whiskey is made from a mash that contains at least 80% corn, distilled at not exceeding 160° proof but, if stored, it must be in oak containers either new uncharred oak barrels or used oak barrels, and bottled at not over 125° proof.

A whiskey may be called a straight whiskey if it is distilled at not over 160° proof and withdrawn from the distillery at not more than 125° proof, aged for at least two years in charred new oak barrels and bottled at not less than 80° proof. The straight whiskey may be identified further as "bottled in bond" if it has been stored for at least four years, bottled at 100° proof and distilled at one plant by the same proprietor.

Sour mash fermentations must have at least 20% stillage (dealcoholized fermented mash) in the fermenter and have a fermentation period of at least 72 h.

Blended whiskey contain at least 20% straight whiskey, on a proof gallon basis (one gallon at 100° proof) either separately or in combination with whiskey or neutral spirits. Depending on the material being blended, whiskey may further be classified as, for example, a blended rye whiskey or blend of stright rye whiskeys.

Spirit whiskey is a mixture of neutral spirits and not less than five percent whiskey, or straight whiskey. If straight whiskey is over 20% it would be classified as a blended whiskey.

Light whiskey, a newly established category, is produced at not less than 160° proof and not more than 190° proof and is stored in used charred oak barrels or new uncharred oak barrels. If light whiskey is mixed with less than 20% straight whiskey on a proof gallon basis, the mixture is designated as blended light whiskey.

Canadian whiskey is subject to Canadian laws and must be at least three years old.

Scotch whiskey is made in compliance with United Kingdom laws. Little information is contained in the regulations. However, the mash must be a cereal grain saccharified by the diastase of malt and the distillate must be aged at least three years.

Irish whiskey, aged at least three years, is produced under the laws of the Northern Ireland or of the Republic of Ireland government.

Technology of Producing Distilled Beverages

There are two main carbohydrate sources used in the distilling industry; those that have to be treated to make the carbohydrates available for yeast fermentation, as in grains, and those that contain fermentable carbohydrates, without further treatment, as in molasses. The two types will be discussed separately.

Distillates from Cereal Grains

There are many distilled beverages produced from grains but, in India malt whiskey is the most typical.

1. Bourbon whiskey

In the manufacture of Bourbon whiskey, the primary concern is to produce a consistent product with a desired flavor. To insure consistency, a manufacturing program is followed which restricts operational variations. A "desired flavor" is defined by individual product managers and varies with each type of Bourbon. Different flavors are obtained primarily by varying the types and percentages of grains used and the selection of a yeast which produces the desired congeners (volatile flavor components). The operation is a science, but there are many unknown influences contributing to slight flavor difference. Thus, two plants, operating under similar conditions, produce distillates with different flavors which experts can readily identify.

a) Raw materials and material handling

The grain bill of a Bourbon mash consists of as little as 51% corn but generally contains 60 to 70% corn. The distiller will generally use a hybrid yellow dent variety because of availability, price, and starch content. An air-dried corn with 14% moisture, 72% starch (dry) and graded as United States No. 2 or better, will produce a good quality product, providing volatile off-flavors are absent. Improperly dried or stored corn, or corn contaminated with pesticides or herbicides will produce poor quality distillates.

In addition to corn, other grains are selected for unique flavors they impart to distillates. These other grains, called small grains, are generally rye, barley and wheat. The most common small grain used in Bourbon production is rye, it contributes a unique spicy and estery flavor. Barley is similar to rye, and the distillates from a corn-barley grain bill have a distinct barley odor without the spicy

character of rye or grainy character of corn. Some Bourbons are made with a wheat grain bill and their distillates have a clean robust flavor. White and Soft Red Winter wheats produce the most desirable distillates.

Almost all the malt used by distillers is made from barley. Distillers generally use malt as a minor component (about 15%) in the grain bill primarily for the enzyme alpha-amylase. Regular distiller's malt consists of thin berries which have a minimum of 55 alpha-amylase units per g of dry malt. In recent years an improved malt has been available to the distiller. It is produced by the standard methods for making malt, except that a solution of gibberellic acid is added to the germinating barley which results in a shorter germination time and almost doubles its alpha-amylase content. The malt standard is based on the alpha-amylase activity but the distiller also relies on the malt to supply beta-amylase and limit dextrinase enzymes as well as to produce a distinct malty flavor in the distillate. The alpha-amylase liquefies the grain mash by randomly cleaving the starch molecule into smaller units (dextrins) while the beta-amylase successively forms maltose units along the starch chain. Both alpha-amylase and beta-amylase attack the alpha-1,4 linkages of starch while the alpha-1,6 linkages are cleaved by the limit dextrinases.

A major factor in selecting a site for a distillery is the availability of an adequate water supply. The properties that are not fully understood, but some odors like mustiness, staleness or medicinal odors will carry over into the distillate and must be avoided.

b) Preparing grain fermentation

The initial step in processing grain involves grinding the entire dry kernel using a hammer mill which has an adequate capacity and is relatively economical to operate. The particle size resulting from grinding is controlled by the size and shape of the screen holes used in the mill and is measured by using a series of sieves having from 0.250 to 1.68 mm openings. A fine grind exposes more of the starch and enzymes in the grain, thus enhancing fermentation efficiencies: while a coarse grind facilitates the recovery of non-fermentable solids after distillation. In practice, the distiller will select a grind best suited to the equipment available: this will represent a compromise between efficient fermentation and by-product recovery.

The proportion of different grains and malt to be used in making a grain slurry, i. e., the grain bill, is determined on the basis of the Bourbon flavor desired. The ground grain and some of the ground malt is mixed with a liquid consisting of water and backset stillage (screened dealcoholized beer). The amount of liquid used to make the meal slurry is controlled by the efficiency of the cookers. A large amount of liquid enhances mixing and heat treating the grain as well as increasing the cost of subsequent operations. The number of gallons of liquid used to cook one distiller's bushel (56 pounds) of meal is designated as the mashing ratio. In processing grain for Bourbon, 20 to 25 gallons of backset stillage and water is used in cooking a bushel of grain.

Preparation of the slurry varies in different plants depending upon the equipment available. In most cases, the liquid portion of the slurry will contain enough backset stillage to produce a slurry having a pH of 4.8 to 5.2. The preparation of the slurry takes place in the vessel used to cook the slurry. The cooker is first filled with the required amount of liquid at ambient temperature and the meal is incrementally added with constant mixing. The grain meal contains 1 to 5% premalt to facilitate cooking by liquefaction of the starch.

When the slurry is heated, it is referred to as mash. Bourbon mashes are processed in a batch cooker by either pressure cooking at 110 to 150°C, atmospherically cooking at 100°C or by infusion at 63 to 69°C. Atmospheric cooking produces a better and a more consistent distillate than either pressure or infusion cooking.

The mashes are constantly agitated while heated with direct steam. Depending on the size of the cooker, grain bill, and mashing ratio, the desired cooking temperature is reached in about two hours. During that time two basic enzyme reactions occur: (1) proteins are degraded to peptides and amino acids which improves yeast metabolism during fermentation and (2) starch is partly degraded by the enzymes from the premalt to promote liquefaction. Starch is susceptible to complete enzyme degradation when it has been adequately heated to cause gelatinization. The temperature required for gelatinization varies with the source of the starch but is always below 100°C. If starch degradation does not occur before the alpha-amylase is inactivated, the mash will form a gelatinous heat transfer from the steam will be difficult and the mash will not be uniformly cooked. Distillates from undercooked mashes will have

a raw grain and pasty odor while overcooked mashers will have a burnt, popcorn, and phenolic odor.

Once the mash reaches cooking temperature, it is held for less than an hour and then cooled to 63°C. The remaining malt of the grain bill, e.g., 8 to 13% (conversion malt) is added and the mash is held for further starch degradation. Conversion malt further liquefies the mash through alpha-amylase activity to prevent gel formation when the mash is cooled to 20°C and to form fermentable sugars through beta-amylase activity. After conversion malt is added and the mash is held for 15 to 30 min, the cooked mash is pumped through a heat exchanger for cooling to 20 °C and then pumped to the fermenter. During the filling of the fermenters, a yeast inoculum is added along with additional backset stillage. The total amount of backset stillage, i.e., the amount added to the slurry for pH control plus the amount added directly to the fermenter, will represent at least 25% of the total volume of material in the fermenter when all the mash, yeast and backset stillage have been added to the fermenter, water is added to bring the total volume in the fermenter to a prescribed level. The content of the fermenter is agitated at which time the fermentation is considered "set".

c) Yeasting

Yeast strains of *Saccharomyces cerevisiae* used to ferment Bourbon mashers are selected mainly for their ability to produce unique congeners in the distillate. Selected strains of yeast will be used for each grain bill as well as for each distillery. These strains produce at least 6% (v/v) of ethanol in a medium containing 11% (w/v) starch. In order to suppress the proliferation of indigenous lactic acid bacteria, a 2% (v/v) yeast inoculum is used.

In current distillery practice, pure cultures of yeast are maintained at 4°C on nutrient agar slants for short-term storage or lyophilized for long-term storage. A large yeast inoculum is prepared from the nutrient agar slant by a stepwise propagation of the yeast insuccessively larger volumes until an adequate inoculum is available to inoculate a plant fermenter.

d) Fermentation

Historically, Bourbon fermenters were constructed of cypress, redwood or larch, but newer facilities may use stainless steel fermenters. The distillates obtained from wood and stainless steel

fermenters have slightly different quality characteristics but no significant differences in preference. Wood fermenters are generally smaller than stainless steel fermenters and require more labor for maintenance and operation. Fermenters are usually cylindrical with a bottom that is sloped toward a drain opening. Ideally, Bourbon fermenters are equipped with agitators to mix the finished beers before dropping to the beer well (temporary storage vessel before distillation). The fermenters may also be equipped with external coolers to control the beer temperature during fermentation.

After 72h, when fermentation is completed, the beer is agitated and transferred to the beer is agitated and transferred to the beer well with minimal aeration to prevent aldehyde formation. The empty fermenter is rinsed with water to remove all grain particles, sanitized with a chlorine solution, and kept hot by introducing live steam to the base of the covered fermenter.

Fermentation is a dynamic process involving a series of reactions: (1) enzymes added after cooking continue to convert the gelatinized starch to dextrins and fermentable sugars, (2) the yeast metabolizes sugars via the Embden-Meyerhof pathway to pyruvate and from there to acetaldehyde and finally to ethanol, (3) the lactic acid bacteria inherent in the mash rapidly increase in numbers and produce acid while the rest of the indigenous population dies off, (4) the yeast and bacteria produce small amounts of a variety of products, some of which are volatile and contribute to the congeners in the distillate.

Most distillers prefer to set fermenters at about 20°C, to control the rate of fermentation so that some activity continues throughout the 72 h period required by regulations and to control the required by regulations and to control the maximum temperature of the fermenter at 32-35 °C. A completed fermentation resulting in inactivity for the last 8 to 12 h is associated with poor quality distillates, and a rapid fermentation resulting in a fermentation temperature over 38°C will inactivate most yeasts and retard the fermentation.

Rate of alcohol production depends upon the set temperature of the fermentation. If fermentations are set at 21°C or below (Fig. 13.5), the initial rate of alcohol production is relatively slow for the first 16 to 20 h. If fermentations are set at 27°C or above alcohol production occurs rapidly and almost half of the alcohol is

produced in the first 24 h of fermentation. After 48 h of fermentation, the slow rate of alcohol production corresponds to the low levels of fermentable carbohydrate left in the fermentation.

Congeners include all compounds in a distillate that affect its aroma and taste. They originate from the grains or are formed during fermentation, distillation.

Although the fermentative yeast is the most important microorganism in the fermentation, lactic acid bacteria are also of importance in producing a high quality distillate. In a well run operation, the mash entering a fermenter has about 100 lactic acid bacteria (predominantly *Lactobacillus* and *Pediococcus* species) per mL. By the time the fermenter is filled and set, the young beer will contain about 1000 bacteria per mL. These bacteria increase in numbers (Fig.13.4) until fermentation is finished when they approximate one billion cell per mL beer.

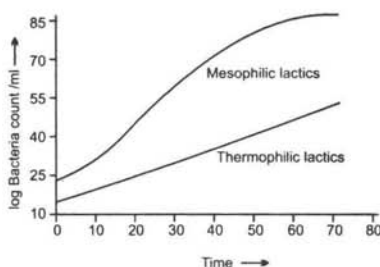


Fig. 13.3 : Profile of lactic acid bacteria during a Bourbon fermentation.



Fig. 13.4 : Exterior view of a distillation column

e) Distillation

Distillation separates, selects, and concentrates the ethanol and congeners from the fermented grain mash (drop beer). Although a great number of different distilling systems are available for Bourbon production, the most common system used is a continuous whiskey separating column, with or without an auxiliary doubler unit. The distillation column (beer still: Fig. 13.7) is a cylindrical shell which is divided into three major sections: **stripping**, **entrainment removal**, and **rectifying**.

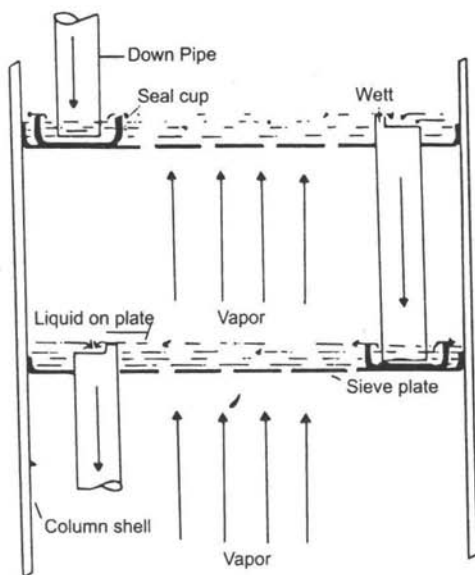


Fig. 13.5 : Cross section of a beer still column showing two stripping plates.

Stripping section: The stripping section contains from 14 to 21 stripping plates (sieve hole plates; Fig. 13.8) which remove alcohol and other congeners from the beer. The perforations are from 1 to 1.25 cm in diameter and take up about 7 to 10% of the plate area. Each plate is equipped with downpipes for transporting the beer to the next plate. There is a chord-shaped weir on the inlet side of the plate to seal the downpipes against the escape of vapor and a second weir on the discharge side to maintain a liquid level on the same plate. The bottom of the downpipe is positioned about 2.5 cm above the surface of the next plate below, so that it is sealed by the liquid level maintained by the weir.

Entrainment removal section: In the entrainment removal section, a plate is installed above the top stripping plate to collect the particulate matter entrained in the vapor.

Rectifying section: The rectifying section consists of three or four rectifying plates (wine plates) above the entrainment removal plates. These are sufficient to yield a product up to 160° proof. To achieve intimate contact between the vapor and the liquid, either

bubble-cap plates (Fig. 13.9) or protruded-hole plates are used as the retifying plates.

A flow diagram of a continuous column distillation system for the production of Bourbon whiskey is shown in (Fig.13.10). Drop beer is pumped at a constant rate to the feed plate and flows across this plate to a downpipe. This cross flow and downward flow pattern continues through successive plates until the liquid reaches the bottom of the beer still.

Direct steam is introduced at the base of the still causing the liquid in the base to boil. The vapor generated flows upward through the sieve holes of each plate. On each sieve hole plate, vapor and beer are well mixed, and heat exchanges between these two phases. The volatile components are vaporize from the beer, and the ascending vapor stream moves to the ascending vapor stream moves to the next plate. As the vapor proceeds successively through each plate, it becomes enriched in alcohol and congeners.

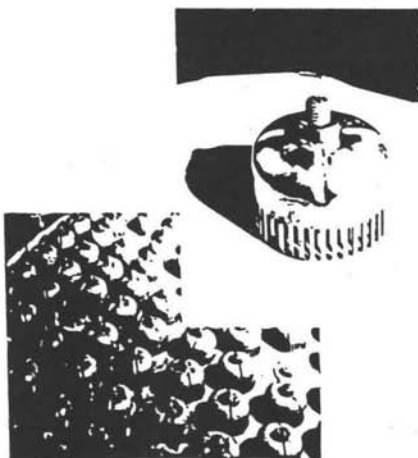


Fig. 13.6 : Photograph of bubble cap plate and individual bubble cap.

As the volatile fractions of the beer are being removed, the remaining fraction (stillage) is continuously being discharged from the base of the still. To produce a high quality distillate, approximately 0.05 to 0.10% (v/v) alcohol should remain in the stillage.

In some stills, the vapors are further refined by passing through a copper mesh demister located in the uppermost section of the column. The volatile sulfur compounds, which give the distillate an undesirable flavor, react with the copper and are thereby removed. The vapor is then partly condensed in a dephlegmator condenser and the remaining vapors flow to a vent condenser where they are further condensed. Non-condensable gases, such as carbon dioxide

and air, dissolved in the beer, and traces of low boiling congeners are permitted to discharge through the vent.

Condensate (Beer still distillate) from the dephlegmator is withdrawn at a specified proof in the range of 110° to 159°. A small portion of this condensate and all of the condensate from the vent condenser are returned to the rectifying section of the column as reflux. This reflux stream may be returned to one of the top three plates. The major portion of the distillate from the dephlegmator is transferred to a separate area (wine room) where it is officially measured for alcohol content (proofing) and total volume (gauging). The distiller will also evaluate the quality of the distillate to make sure it is worthy of being barreled.

Before the wine room operation, some distillers redistill the product in a simple copper pot still (doubler kettle) heated by steam coils and condensing the vapors in a copper condenser. This gives a somewhat "cleaner" flavor by removing some of the high boiling congeners from the product.

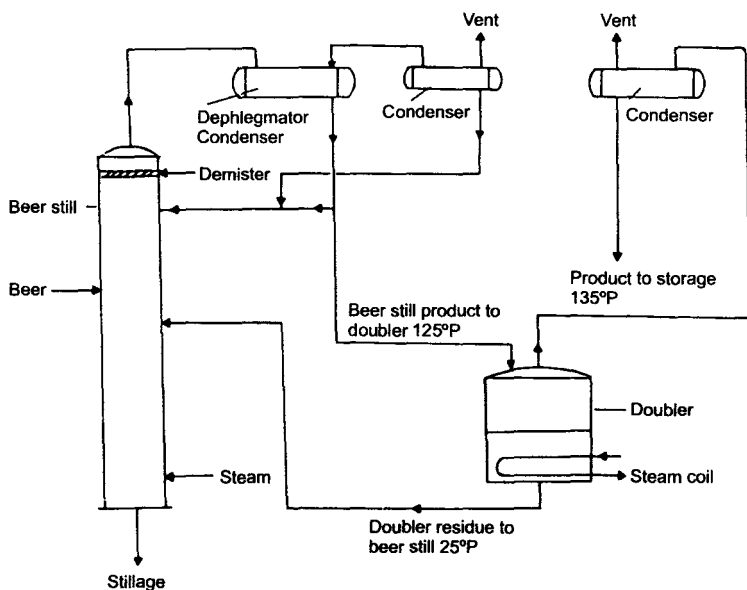


Fig. 13.7 : Schematic drawing of a whiskey beer still and doubler.

In the doubling process, the beer still distillate is reduced to 90 to 100° proof and charged into the doubler kettle. When boiling

begins, a heads fraction is withdrawn from the condenser. A product fraction is withdrawn until alcohol concentration in the kettle is practically zero and the tails fraction is retained in the kettle. The product obtained has reduced congeners of low boiling heads (aldehyde) and high boiling tails (grainy, sloppy). The heads and/or tails can be redistilled with the next charge.

f) By-product recovery

Whole stillage, the product discharged from the base of the whiskey column, is composed of substances derived from grain as well as yeast cells and various nutrients formed during the fermentation. Whole stillage, containing 5.8% suspended solids, is pumped over screens to separate the coarse material from the liquid. The screen cake, at 80-85% moisture, is fed to presses which further reduce the moisture to 57-69%. The press cake is then drum-dried to 10% moisture. This dried product is called Distiller's Dried Grains. The liquid portion (thin stillage) from the screens and presses is collected and fed to a multiple effect evaporator which concentrates the liquid at 4-5% solids to syrup of 25-45% solids which is metered into the press-cake before it enters the drum dryer. The mixture containing syrup and press-cake with some recycled dried product is dried to 10% moisture. The finished product is called Distiller's Dried Grains with solubles. These dried products are used to fortify dairy, poultry, and swine feeds, Distiller's feeds are rich in proteins (24-35%), fat (8%), choline, niacin and other B-vitamins various minerals, and other vital growth factors for livestock and poultry.

g) Distillate processing

The product of distillation requires further processing before being bottled. The Bourbon is barreled and sent to a warehouse for maturation. The progress of maturation is evaluated to determine when the Bourbon is mature. When mature, the barrel contents are emptied, screened, filtered and bottled. These various steps will be described in detail in the following sections.

Maturation

The last step in Bourbon production is the maturation process. The Bourbon distillates at 105° to 125° proof are put into new, charred, white oak barrels. The fill hole is closed with a poplar wood bung and the barrels are entered in a warehouse.

Barrels lose liquid volume during storage for several. The main loss, four percent per year, is due to evaporation. After five years of storage, about 10.5 proof gallons are lost. A smaller loss, about 3 proof gallons, results from soakage into the wood.

The optimum temperature for maturation ranges from 21 to 30°C, which results in a Bourbon with the desired flavor. If the temperature increases much over 30°C, the Bourbon flavor becomes too woody and resinous. At high temperature and low humidity, water migrates through the barrel staves at a faster rate than either ethanol or fusel oil. Therefore, the percentage of these alcohols increases. The converse is true when a warehouse. The converse is true when a warehouse is cold and damp.

Changes in the whiskey during storage are caused by three types of reactions: (1) chemical interactions between the distillate and charred wood: (2) chemical interactions among components of the distillate: and (3) physical extraction of barrel compounds. An example of interaction between the distillate and charred wood is the reaction between alcohol and lignin, forming an ethanol lignin compound which breaks down during maturation between constituents of the distillates would be the reaction to form congeners such as aromatic aldehydes, e.g., vanillin. An example of interaction between ethanol and acetic acid to form ethyl acetate. The third reaction is typified by the extraction of non-volatile dissolved solids which give color or body to the product.

Blending

Each lot of Bourbon is periodically checked for its maturation characteristics such as smooth taste and pleasant aroma. The rate of maturation depends on the grain bill, geographical location of the warehouse, and the location of the barrels within the warehouse. After the Bourbon has been judged to be acceptable, it is ready for withdrawing from the warehouse.

If a blended Bourbon is required, a trial blend is made in the laboratory. Once the blend is identical to its flavor standard, the proper number of barrels of each lot are withdrawn and the barrels emptied. The whiskey is screened to remove barrel char. The whiskey is then pumped to a gauging tank where the proof gallons are then blended based on the laboratory formula. The blends are reduced to a few tenths of a point above the desired proof by adding demineralized water. The blend is allowed to "marry" i.e., it is held

for a time to allow it to become completely mixed and smooth. It is then chill filtered, refiltered and sent to a bottling tank.

Filtration

Bourbon whiskey after maturity contains two types of insoluble substances, noncompressible and compressible, which must be reduced or removed to achieve satisfactory clarity. Failure to reduce or remove these materials results in formation of either "cold cloud" floc, puff balls, sediment or haze. These conditions occur more frequently when the whiskey is stored for extended periods at reduced temperature. The non-compressible substance, such as char and crystalline matter, originate from the barrel. The compressible substances are ethyl palmitate formed during fermentation and carried over in the distillate, and betasitosterol and beta-sitosterol-D-glucolide which are leached from the barrel during maturation. These compounds are less soluble at low proof and low temperature. To facilitate their removal, the temperature is lowered to - 9°C to cause these compounds to precipitate and they can then be removed by filtration. This procedure gives improved clarity and stability if the product is subjected to low temperatures either in shipping or storage.

Many types of filter equipment are used for whiskey filtration such as bag, vertical tank, horizontal plate, plate and frame, pressure leaf, sealed disk cartridge and spool cartridge. Filter materials used with the above equipment include combination of cellulose and diatomaceous earth, woven natural or synthetic materials and membranes.

In summary, primary filtration removes barrel char solids, chill filtering removes the compressible solids; a secondary filtration polishes the whiskey; and a final filtration ensures product clarity before packaging.

Neutral Spirits

The primary objectives for manufacturing neutral spirits in India are to produce alcohol in a cost effective manner and to obtain a distillate essentially free of any congeners. To achieve these objectives, the processing methods differ significantly from Bourbon production where flavor development is of prime concern.

Corn is the grain of choice for manufacturing neutral spirits although other grains are used if they are more economical. Milling

and mashing procedures are similar to those used in making Bourbon except that the ratio of liquid to ground grain in the slurry is reduced as low as is operationally practical.

The cooking procedure eliminates the premalting step by using a continuous system in which a jet heater rapidly raises the temperature of the corn slurry past its gelatinization temperature to either 100 °C (atmospheric cooking) or 160 °C (pressure cooking). Holding time at the cooking temperature is controlled by the plug flow of mash through the systems at a predetermined rate. The mash is cooled to its conversion temperature by vacuum if it is atmospherically cooked or by flashing to atmospheric pressure and then by vacuum if pressure cooked.

There are several methods available to liquefy and saccharify the starch in the mash. If backset stillage is added to the corn slurry, and there is a two to five minute holding time between the last vacuum chamber and the mash coolers, a fungal alpha-amylase preparation is added to the cooked mash at 6.3°C. If no backset is added to the corn slurry and/or a holding time with mixing for 30 min or more exists between the vacuum chamber and the mash cooler, a thermal stable bacterial alpha amylase is added at 88°C for liquefaction.

The temperature at which spirit beer mash is set depends on the efficiency of the beer coolers. Ideally, a fermentation is set at 32°C and controlled at 38°C. The yeast strains used for fermenting corn mashes differ from strains used for fermenting Bourbon mashes. The criteria for selecting neutral spirit yeasts include alcohol production, alcohol tolerance, rate of alcohol production, and temperature tolerance. Yeasts in current use are capable of producing about 14% (v/v) alcohol after 60 h of fermentation.

Finished beer is distilled in a beer still and further processed to remove essentially all of the congeners present. The types of additional distilling columns used depend upon the amount of heads and tails fractions in the product. The most common systems used in the distilling industry are: (1) the continuous four-column system for the production of grain neutral spirits; and (2) the batch rectifying column and kettle unit used primarily in the production of grain neutral spirits that are subsequently stored in barrels for maturation.

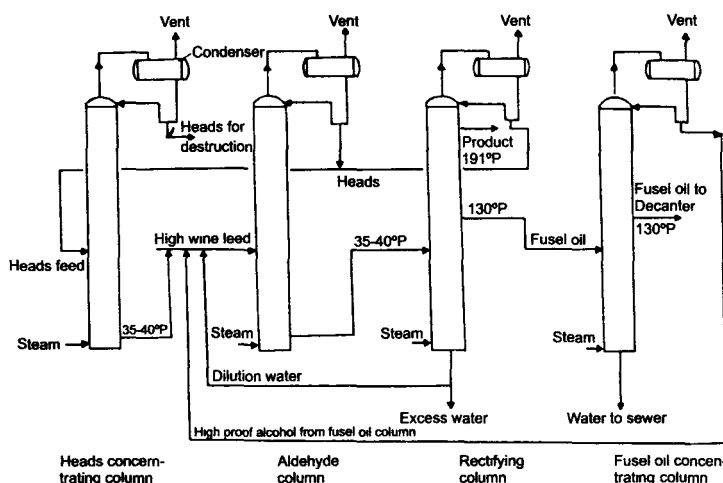


Fig. 13.8 : Flow diagram of four column unit for continuous distillation of neutral spirits.

The four-column continuous distillation system (Fig.13.8) for production of neutral spirits consists of aldehyde, rectifying, fusel oil concentrating and heads concentrating columns. The distillate is purified in the former two columns while the congeners are concentrated and removed using the later two columns.

Aldehyde column: Beer still distillates and recycle streams from other columns are refined in this column. Product is removed from the base at 35 to 40° proof, which is controlled by the addition of dilution water to the feed. The dilution water comes from the base of the rectifying column. The product is then transferred to the rectifying column. Heads containing low boiling congeners at 193° proof are removed from the condenser, and fed to the heads concentrating column. A portion of the heads condensate is returned to the top plate of the aldehyde and rectifying columns as reflux.

Rectifying column: This column concentrates the alcohol in the feed from the base of the aldehyde column to the desired product proof. It also removes fusel oil, intermediate and high boiling esters, acids and phenolics. In addition, some of the low boiling heads present in the feed may be removed from the product. The product at 192 to 192.5° proof is collected from the condenser or may be removed as a side draw-off from a point 5 to 7 plates below the top.

This latter operation makes it possible to take a 3 to 5% heads cut, giving a more neutral product. Fusel oil is removed from the side of the column at 130° proof. This draw-off also contains phenolics, high and intermediate boiling esters, and traces of high boiling acids. Water which contains the grainy and sloppy congeners and traces of acetic acid is discharged from the base. A portion of the water is used for dilution purposes in the aldehyde and heads concentrating columns and the remainder is discharged to the sewer.

Fusel oil concentrating column: Fusel oil, high and intermediate boiling esters and phenolics in the feed stream are removed in this column. Alcohol carried in the feed is reclaimed from the condenser and recycled to the aldehyde column. The recovered alcohol should contain only trace amounts of fusel oils. The fusel oil is removed from the plate in the column at 130° proof, which is optimum for the concentration of these congeners. This stream is diluted to 30 to 40° proof with water obtained from the base of the rectifying column and the mixture is transferred to a decanter. Here, an oil layer collects at the top and an aqueous layer at the bottom. The oil layer is withdrawn as concentrated fusel oil and the aqueous phase is returned to a feed plate of the fusel oil concentrating column. The fusel oil is collected in a storage tank.

Heads concentrating column: The principal functions of this column are to separate and remove low boiling esters, aldehydes, acetals, diacetyl, and methanol from the feed stream and to reclaim the ethyl alcohol in the feed for eventual recovery as product. This column receives streams from the aldehyde and rectifying columns. A sufficient quantity of water obtained from the base of the rectifying column is added to the feed stream to give 35 to 40° proof out of the base. The base alcohol stream, essentially free of heads, is recycled to the feed plate of the aldehyde column. Heads are withdrawn from the condenser and are transferred to a storage tank for eventual disposal as "heads-for-destruction". A portion of the condensate is returned to the top plate as reflux.

The batch rectifying column and kettle is operated batchwise for the production of neutral spirit. The column normally consists of 50 to 60 plates, which reduces the percentage of heads and tails draw-off required to give satisfactory product quality. The kettle is sized in proportion to the column diameter such that the amount of ethyl alcohol retained on the plates (column inventory) is about

8% of the kettle. Condensing equipment consists of a dephlegmator condenser, a vent condenser and a product cooler.

The distillate is fractionated into heads, product, and tails fractions. The high boiling congeners such as fusel oil, acids and phenolics either remain in the kettle or are removed in the tails fraction. However, some congeners present in trace amounts will distill off with the product fraction because of their similar volatility. For any given column and kettle system, the percentage of heads and tails that is removed to give a satisfactory product quality is determined by trial and error. Typically 3.5% of the alcohol in the high wine is withdrawn as heads at a reflux ratio of 10:1 (10 volumes of reflux to 1 volume of draw-off) and 86% is withdrawn at 192 to 192.5° proof as spirit at a reflux ratio of 4.5:1. The product is cut over to tails when the base temperature reaches 102°C.

Other whiskeys

The production of whiskeys other than Bourbon that are of economic importance will be discussed individually.

a) Scotch whiskey

There are two basic types of Scotch whiskey, namely, malt whiskey and grain whiskey.

The majority of scotch whiskeys sold are blends of malt and blends of malt whiskeys are also produced. The blending formulae are trade secrets of the blender.

An analysis of a blended scotch whiskey is shown in Table 13.1. The production of the two basic types is described below.

Table 13.1 : Congener Analysis of Scotch, Irish and Canadian Blended Whiskeys*

Congeners	Whiskey, g/100 L at 80° proof		
	Scotch	Irish	Canadian
Total acids	28	10	16
Ethyl acetate	22	6	10
Total higher alcohols	102	49	31
n-Propyl	20	25	2
Isobutyl	34	15	7
(+)-Amyl	48	9	22
Acetaldehyde	6.4	1.6	1.8
Furfural	0.7	0.2	0.4

Malt whiskey. Malt whiskey is made from peated barley malt as the only source of starch for the production of malt whiskey. The enzymes in the malt change the starch to sugar during the mashing process.

Malting in the process of germinating and drying barley for amylase production. The barley is saturated with water in steeping tanks, drained, and under proper conditions, germinates and subsequently sprouts. The sprouting grain must be turned over daily: this keeps it aerated and prevents it from matting. This green malt is then kilned. It is spread on a perforated floor and smoked by burning peat below the malt. The smoke from the peat, called peat reek, is absorbed by the malt which gives the whiskey its unique flavor.

The level of peating is measured by the phenol content, which varies from 2 to 20 ppm. After the smoke treatment, the malt is dried at 70°C to stop any further growth. The dry acrospires or rootlets, called culms, are then removed and sold for cattle feed. The barley malt is then crushed in a roller mill, and the resulting malt particles are called grist. The size of the grist has to be closely controlled. A coarse grist impedes starch conversion: too fine a grist clogs the mash tun and slows the drainage of the wort.

The mash is prepared by mixing grist and liquid at 65 to 70°C at a ratio of 1 kg to about 3.2 L of liquid. The liquid is composed of the last two washings of the previous mash. The mash is well mixed during the filling operation and then is held for one hour at 64°C for the amylolytic enzymes in the malt to change the starch to maltose. The mash tun is drained and the wort is cooled to 21°C and sent to the wash back and the mash is pitched (yeasted) with commercial yeast or yeast propagated at the distillery.

The residue on the plate is washed with 76°C liquid, rested and again drained, cooled and pumped to the wash back. This procedure is repeated twice more with hotter water and the washings are sent to the hot water tank to be used as liquid for the next mash. The draff (spent grain) is removed from the tun, and sold as cattle feed.

Scotch fermentation yield 10% alcohol, v/v, and are complete in about 36 h because most of the carbohydrates are converted to fermentable sugars during mashing and are therefore immediately available to the yeast. Since the wort is not heated to a pasteurizing

temperature, a variety of bacteria remain viable, although only the lactic acid bacteria continue to multiply from a few million to several billion per mL under normal fermentation. Each distillery seems to have its own indigenous bacterial flora, which has a bearing on the flavor of the whiskey because of the varied end products they produce.

Malt whiskeys are made by distilling wash (fermented wort) in a wash still, a type of pot still. The wash is pumped to a wash still. The distillate, a low wine of about 41° proof, is sent to the low wine still to remove the undesirable components and raise the proof to 140° to 160°. The foreshots (heads) are distilled off and sent back to the wash still. The whisky is drawn off and the feints (tails) are also scut back to the wash still. The residue remaining in the wash still (pot ale) is drained and then converted to animal feed.

The pot still distillates are reduced to about 110° proof and matured in used oak casks for at least three years. When judged to be mature, they may be bottled as malt whiskeys or blended with grain whiskeys and rebarrelled for "marrying" before bottling.

Grain whiskey. The production of grain whiskey in Scotland is similar to the production of light whiskey (see below) in the United States, except that Scotland is not permitted to use microbial enzymes for saccharification.

The whiskeys are produced at 180° to 186° proof using a Coffey still, and aged for a minimum of three years in used oak casks prior to blending, generally with grain whiskeys.

b) Irish whiskey

There are two types of Irish whiskeys: pot still and grain. Pot still whiskey is made from a grain bill consisting of barley malt, barley, and small quantities of wheat and rye. Grain whiskey is made from a grain bill consisting mainly of corn. Mashing and fermentation procedures are similar to those used in Scotch production. Irish pot still whiskey is triple pot distilled to 140 to 150° proof: grain whiskey is distilled on a multicolumn unit. The results of the analysis of a typical Irish blended whiskey are shown in Table 13.1.

c) Rye whiskey

The technology for manufacturing rye whiskey is essentially the same as that used for Bourbon whiskey. The mashing procedure

involves atmospheric cooking at or near 100°C or infusion at approximately 70°C. Rye distillates are used primarily to obtain unique flavors when mixed with other types of whiskey. Some are marketed as straight rye whiskey.

d) Corn whiskey

Corn whiskey is prepared in a manner similar to Bourbon, the major difference is the type of cooperage used for maturation. Corn whiskey is aged in used Bourbon barrels or new uncharred oak barrels, whereas, Bourbon is aged in new charred barrels. Corn whiskey is lighter in flavor than Bourbon. The finished product is usually not sold as corn whiskey and it is used primarily as a blending item. For a comparison of unaged and aged corn and Bourbon whiskey, see Table 13.2.

Table 13.2 : The influence of Aging on Corn and Bourbon Whiskey

Congeners	Whiskey, g/100 l. at 100° Proof			
	Corn		Bourbon	
	Unaged	Aged, 4yr	Unaged	Aged, 4yr
Total acids	0.6	1.9	0.9	50
Ethyl acetate	7	15	6	34
Acetaldehyde	0.6	2.7	0.6	3.7
Fusel oil	179	185	202	218
Furfural	0.80	0.30	0.03	1.0

e) Light whiskey

The procedural technology for manufacturing light whiskey is basically the same as that described above for neutral spirits, except that light whiskey is distilled between 160 and 190° proof. This whiskey was produced to meet the shift in consumer preference toward the lighter flavored products.

f) Blended whiskey

Blending is the process of combining different whiskeys according to predetermined proportions so as to obtain a product with the desired flavor and balance. Blended whiskey is prepared from Bourbon, rye corn, and light whiskeys. The blending and filtration procedures are essentially the same as previously discussed.

A comparison between an American blended whiskey and a Bourbon whiskey is shown in Table 13.3. The blended whiskey contains lower levels of ethyl acetate, total higher alcohols (fusel oil), and aldehydes than Bourbon whiskey.

Table 13.3 : Congener Analysis of American Blended and Bourbon Whiskey

<i>Congeners</i>	<i>Whiskeys, g/100 L, at 80° proof</i>	
	<i>American Blended</i>	<i>Bourbon</i>
Total acids	30	58
Ethyl acetate	17	38
Fusel oil	55	162
n-Propyl	3	11
Isobutyl	12	38
Amyl	40	112
Acetaldehyde	2.7	5.2
Furfural	0.36	0.98

g) Canadian whiskey

Canadian whiskey, produced under the regulations of the Canadian government, is made from a mash of cereal grain and produced solely within Canada. There are no restrictions as to grain bills, distillation procedures, or cooperage, but the respective procedures used are basically the same as those in the United States.

Most of the Canadian whiskey sold is a blend of approximately 90% neutral grain whiskeys and 10% Bourbon or rye type whiskeys. The results of the analysis of a typical Canadian blended whiskey are shown in Table 13.1.

Distillates from Non-Grains

There are a number of distilled beverages made from substrates other than grains. Three of these are discussed below.

1. Rum

Rum is produced by distilling fermented molasses, a by-product of cane sugar refineries. The molasses is diluted with water to obtain a mixture containing 12 to 20% sugar, and adjusted to pH 4.0 with sulfuric acid to control bacterial contamination during fermentation.

Molasses contains most of the nutrients required for fermentation except for nitrogenous compounds (Table 13.4) which are obtained by adding either urea or ammonium salts of Phosphate or sulfate. The fortified molasses is inoculated with 4 to 10% yeast (v/v) propagated in a molasses medium. The initial fermentation temperature is around 21°C and reaches a maximum of 33°C. For light flavored rum, the fermentation time is 1.5 to 2 days. For the production of heavy flavored rum, the less from cane juice and calcium carbonate are added. The fermentation requires 12 days for completion. Additional factors which influence the flavor of rum include sugar cane variety, conditions during the growing season, geo-graphical location, yeast strain and distillation techniques.

Rum distillation involves the use of three units, namely, a beer still, an extractive column and a rectifying column. A schematic representation of the latter two processes is depicted in Fig. 13.8. The product is collected at 180 to 190° proof and aged in oak casks until the desired flavor is obtained, usually one

Table 13.4 : Physical and Chemical Composition of Sugar Cane Molasses

Brix density	89 ^a
pH	5.30
Apparent purity	28.67
Sucrose, % (w/w)	33.78
Reducing sugar, % (w/w)	26.96
Total sugar, % (w/w)	60.94
Sulfated ash, % (w/w)	9.46
Gums, % (w/w)	N.D.
Total nitrogen, % (w/w)	N.D.
Minerals in ash, % (w/w)	
Potassium	2.68
Magnesium	0.98
Calcium	0.88
Sodium	0.06
Boron, ppm	410
Iron, ppm	158
Manganese, pp	57
Copper, ppm	28
Zinc, ppm	11
Nickel, ppm	1
Lead, ppm	0.75
Cobalt, ppm	0.54

Table 13.5 : Congener Profile of Aged Light and Dark Rum at 80° Proof (g/100 L at 80° proof)

Analysis	Light Rum	Dark Rum
Total solids	400	300
Total acids	6	14
Ethyl acetate	3	10
Higher alcohols	2	20
Acetaldehyde	0.2	4
Furfural	0.02	0.1
Acetal	0.4	1.0
Tannins	2	7

^aTaken from in-house publication

^b As acetic acid

to two years. Heavy flavored rum is manufactured by distilling a 12 day old fermented molasses in a pot still where the "heads" and "tails" cuts are recycled in successive distillations. The distillate is matured for a period of approximately three years in oak casks. A typical congener profile of an aged light and dark rum is presented in Table 13.5.

2. Vodka

Unlike whiskeys which must be produced from grains, vodka can be produced from any carbohydrate source (white potatoes, cassava, jerusalem artichokes, sugar cane or sugar beets). The carbohydrate is extracted from the plant material by whichever means are appropriate. The fermentation and distillation technology for vodka production is essentially the same as that discussed above for neutral spirits.

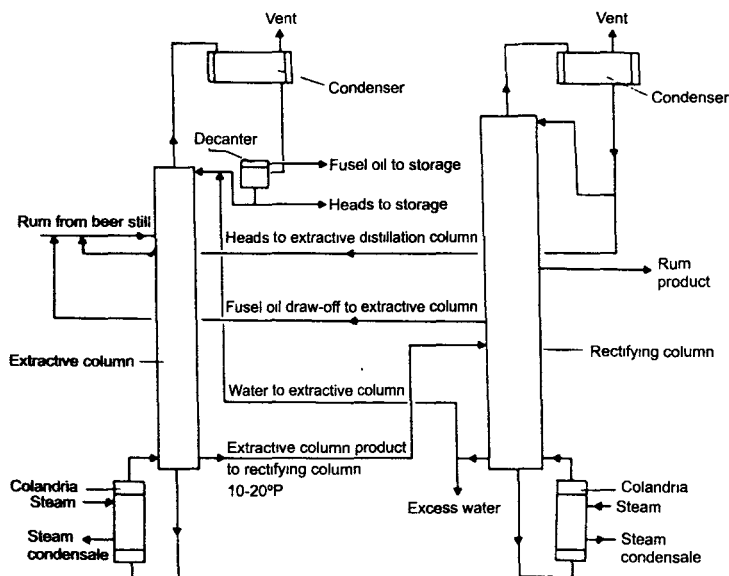


Fig. 13.8 : Schematic diagram of extractive and rectifying columns for rum distillation.

3. Tequila

Tequila, an alcoholic beverage manufactured in Mexico, is made from a cactus plant, *Agave tequilana*, Weber. Cacti are harvested when they are approximately 13 years old. Their leaves and stems

are removed, leaving a bulbous core of tissue weighing approximately 35 kg. The core is chopped and cooked in a masonry oven with direct steam. The cooking time, at pH 5 to 6, will vary from 9 to 24 h, depending upon the rate at which inulin is converted to fermentable sugars. After cooling, the cooked tissue is

Table 13.6 : Congener profile of white (Unaged) and Aged Tequila at 80° proof

<i>Test</i>	<i>Unaged (g/100 L)</i>	<i>Aged</i>
Total solids	4	40
Total acids ^{b)}	11	34
Ethyl acetate	4	19
n-Propyl	18	24
Isobutyl	7	10
Amyl	35	41
Acetaldehyde	4.3	6.3
Furfural	0.23	0.64
Acetal	2.7	3.7
Tannins	1	6

processed through a shredder and a roller mill to separate the juice from the pulp. The pulp is washed once with water to remove more sugar for fermentation. The juice can be supplemented with sugar cane syrup and/or brown sugar not to exceed 30% of the total fermentable sugar in the fermentation. Ammonium sulfate is added to the juice at a ratio of 1:287. Sufficient water is added to the fortified juice to bring the sugar content to approximately 9% (w/v). A 6% (v/v) yeast inoculum is added to the fermentation. The temperature of the 38 to 42 h fermentation may peak at 36°C from the initial temperature of 30°C. The alcohol concentration of the finished fermentation is about 4% (w/v) and the alcohol is removed by primary distillation in a copper pot still constructed with steam coils and condensers. Fresh bagasse is added to the pot still to enhance the characteristic flavor of the final product. The primary distillate is collected at 28° proof and redistilled in another pot still without refluxing to increase the proof to 106°. During this distillation the more volatile compounds are recycled through the first pot still. A flow diagram showing the various processes of a tequila distillery is shown in Fig. 13.9. The product resulting from this distillation may be bottled without further processing as "white" tequila, or matured in oak barrels for at least one year and bottled as aged tequila. The congener profile of an aged and unaged tequila is summarized in Table 13.6.

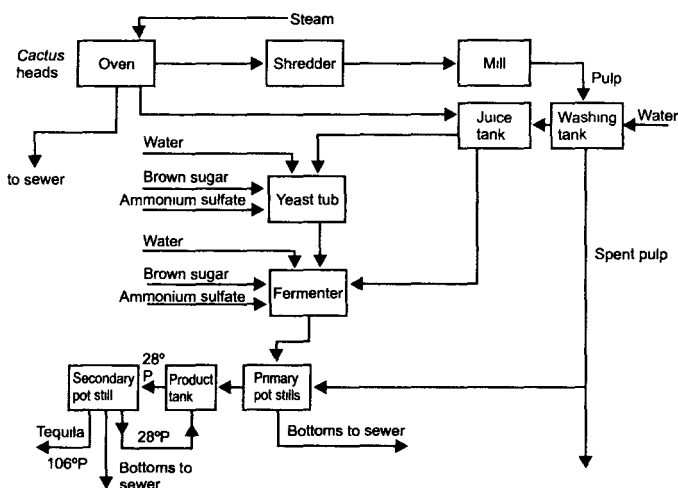


Fig. 13.9 : Flow diagram of tequila production.

Microorganisms Involved in Distilled Beverage production

A. Yeast

Yeasts are often designated on the basis of their respective uses, e.g., a wine yeast is used to produce wine, a baker's yeast for bakery goods. Distilleries have their preferred yeast strains, usually *Saccharomyces cerevisiae*, which give the product the desired properties. These *S. cerevisiae* strains are selected for their consistent ability to produce the desired congeners and the yeasts are classified accordingly. A Bourbon yeast, for example, must grow in a grain mash, produce approximately 6 to 8% (v/v) ethanol with the desired congeners in 72h; a rum yeast must possess osmophilic characteristics and utilize sucrose; and a neutral spirit yeast must efficiently produce ethanol with low congener levels from carbohydrates. The common role of all distillery yeasts is to utilize the available carbohydrate for the production of ethanol.

1. Alcohol production

As previously mentioned, carbohydrates originate from a variety of sources and may not be initially available for yeast metabolism. Initial carbohydrate analysis via high pressure liquid chromatography of a bourbon and a spirit mash is shown in

Fig. 13.10. For a Bourbon mash, approximately 95% of the total starch initially been converted into soluble dextrans, maltotriose, maltose, and glucose. The dextrin and remaining insoluble carbohydrates are then enzymatically converted into maltose and glucose which can then be taken up by the yeast via facilitated diffusion and the permease system.

Facilitated diffusion rapidly transports glucose into the cells without using metabolic energy. Maltose is transported into the cells, via specific carrier proteins called permeases, where it is hydrolyzed into 2 molecules of glucose. These permeases are substrate specific and require metabolic energy for operation. Intracellular glucose is metabolized through the Embden-Meyerhof pathway of glycolysis. The overall reaction is:



Two moles of ATP are also produced which is used to supply energy for cell maintenance and growth. Detailed mechanisms by which the fermentable sugars are taken up by the cell and its metabolism are discussed.

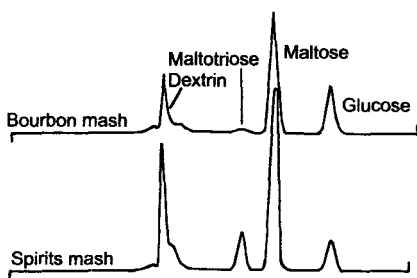


Fig. 13.10 : Carbohydrate profile of set samples of Bourbon and spirits mash.

Theoretically, conversion of a gram of glucose via fermentation yields 0.511 g of ethanol. This theoretical value is never obtained during fermentation due to carbohydrate utilization for cell maintenance, growth, and formation of small amounts of glycerol and higher alcohols. Fermentation efficiency also depends on factors such as yeast strain and environmental parameters. In practice, efficiencies of slightly more than 90% are obtained.

An increase in temperature, within a certain range, increases reaction activity. During a normal fermentation, heat is produced

from active yeast growth and metabolism which causes a rise in temperature. This temperature rise can drastically effect yeast metabolism and ethanol production. An average upper limit temperature for *S. cerevisiae* growth is around 40°C with an optimum temperature of around 30°C. Increased heat tolerances can be obtained with media containing ergosterol and oleic acid. A typical temperature cycle for a Bourbon fermentation may range from 20 to 32°C while a spirit mash may be held at 30 to 32°C for the entire fermentation.

The maximum concentration of ethanol that a yeast can produce depends upon the yeast strain used. As a general rule, yeast cell growth is inhibited around 10 to 12% (w/v) ethanol while 20% ethanol will terminate cellular metabolism established an early basis for alcohol tolerance and reported decreased alcohol tolerance with increased glucose tolerance. High alcohol tolerant strains were also found to store less lipids and carbohydrates than less tolerant strains.

Under "rapid fermentation", increased intracellular alcohol concentration lowered cell viability. They also demonstrated that 9.4% (w/v) ethanol produced via fermentation was more toxic to the yeast cell than 13.8% (w/v) ethanol added to the medium. Higher temperatures are also conducive to higher intracellular ethanol concentration. It thus appears that conditions favoring higher intracellular ethanol also reduce cell viability. A better understanding of the mechanism of ethanol movement through the yeast cell membranes is needed. With newer yeast genetic techniques, it is conceivable that alcohol equilibrium might be changed by alteration of the cell membranes.

2. Congener production

After a yeast strain has been found that accomplishes the task of producing ethanol efficiently under plant operating conditions, the major criterion in yeast selection must be the production of desirable congeners.

Congeners originate from raw materials and/or are formed during mashing, fermentation, distillation, and maturation. Currently defined, congeners include fusel oil (isoamyl, amyl, isobutyl and n-propyl alcohols), aldehydes, esters, and furfural. Flavor contribution to the final product depends not only on

concentration of these components but more importantly on the ratio among these components.

Many factors have an affect on congener production. Their results indicated that higher congener levels were produced from increased inoculation, agitation, or temperature. Generally, congener levels increase as the fermentation rate increases.

Proposed metabolic pathway to explain congener synthesis in yeast is shown in Fig. 13.11. Radioactive labelled glucose and amino acids in Bourbon mash were used in this study. Fusel oils were produced from amino acid and carbohydrate metabolism. These fusel oils were produced mostly from both amino acids during the early part of the fermentation and from amino acids and carbohydrates during late fermentation. In Bourbon fermentations, a range of fusel oil concentration from 80 to 460 g per 100 liters at 100° proof of distillate can be obtained with different distillery yeasts.

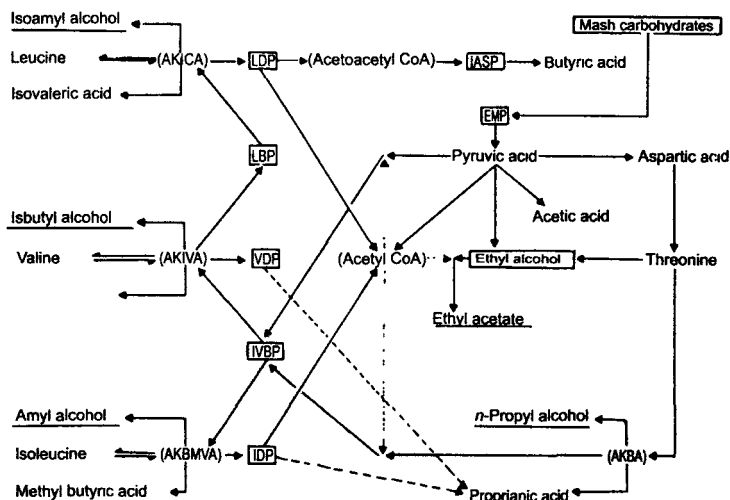


Fig. 13.11 : Proposed metabolic pathway for the synthesis of whiskey congeners.....synthetic and degradative pathways:-----
hypothetical pathways: LBP leucine biosynthesis: IVBP isoleucine valine biosynthesis: FASP fatty acid biosynthesis: LDP leucine degradation:VDP valine Degradation: IDP isoleucine degradation; EMP Embden-Meyerhof-Parnas pathway; PA pyruvic acid; AKBA α -ketobutyric acid; AKBMVA α -keto β -methyl valeric acid: AKIVA α -ketoisovaleric acid; AKICA α -ketoisocaproic acid.

Table 13.7 : Chemical Analysis of Whiskey During Maturation

Age (Months)	Proof	Color	pH	Acids		Alde- hydes	Congeners, g/100L		
				Fixed	Volatile		Esters	Fusel Oil	Ehanol Lignin
0	110.0	0	-	0.26	2.12	0.7	6.87	207	0
6	112.0	147	-	4.10	25.63	3.37	17.68	226	13.6
12	110.0	167	-	5.44	36.15	2.67	21.55	250	27.1
18	111.9	195	-	10.51	41.52	5.15	27.44	228	25.3
24	112.6	215	4.3	12.98	45.03	4.96	33.15	240	41.9
30	111.5	251	4.1	10.95	56.72	5.87	38.22	243	35.7
42	119.0	248	4.0	14.36	49.66	8.25	51.83	249	33.9
56	125.0	270	-	-	-	9.23	57.20	266	-

Congener concentrations and ratios can be altered by fermentation, distillation, and maturation.

Bacteria

Bacteria are involved in a grain fermentation in two widely divergent ways.

1. Lactic souring of yeast mash

Lactic souring is a process in which lactic acid producing bacteria lower the pH of mash used to propagate yeast. The souring process creates an environment inhibitory to bacterial spore germination and to the growth of vegetative bacterial cells. The United States government requires that yeast used for the production of sour mash whiskey must be grown in soured yeast mash. Although there are no such regulatory requirements for other whiskey or spirits, souring is still practiced because of its inherent usefulness for bacterial control.

One method for lactic souring utilizes the native bacteria present on the conversion malt. Natural souring, however, leaves much to chance since there is no consistency or uniformity in the composition of the melt bacterial population. With much emphasis today on quality assurance, souring with a pure culture is a standard practice in the industry. A strain of *Lactobacillus delbrueckii* is an excellent choice because it: a) has a high optimum growth temperature (ca. 50°C) which inhibits the growth of mesophilic and psychrophilic bacteria; b) produces a large amount

of acid within a relatively short incubation time; and c) produces no volatile end products to affect distillate quality.

The mash made from the same grain bill as used for fermentation may be soured for yeast growth. If a comparatively high yeast count is desired, the distiller may prepare the yeast mash with 50% barley malt and 50% rye. For souring, the temperature of the mash is adjusted to 50°C and treated as follows: (1) inoculate with a 0.2 to 0.4% (v/v) inoculum of a 16 to 18 h culture of *L. delbrueckii*; (2) incubate at 50°C until the pH is 3.8 to 4.0 or until an increase of 10 to 13 mL of acid is obtained (mL of 0.1 N NaOH required to titrate 10mL of yeast mash to pH 8.3); and (3) pasteurize at 86°C for 30 min. The pasteurized mash cooled to approximately 28°C is ready for yeast inoculation.

2. Indigenous microflora in the fermentation

The microbiology of the raw materials, equipment, process and fermentation will be discussed individually, with particular reference to distillate quality.

Grain. Conditions which contribute to microbial development on corn include excessive moisture, faulty drying and an excessive number of cracked kernels, which exposes the hygroscopic endosperm to microbial attack. Corn which is heavily contaminated with mold, e.g., may have a musty odor which will carry over and adversely affect distillate quality. The kind of microorganisms present on corn include wild yeast, mold, and aerobic and anaerobic bacteria. Microorganisms on the small grains include the lactic acid bacteria and the same general types associated with corn. Grains as received from the suppliers will have a standard plate count of several million colony forming units per gram.

Cooking and starch conversion. In a typical mashing procedure where the cooking temperature is at or near 100°C, all of the vegetative bacteria cells, yeast and mold microorganisms will be killed but some bacterial spores will survive this temperature and not all microbially-induced odors will be destroyed. The cooked mash is cooled to 63°C and uncooked malt is added to induce starch conversion. This addition introduces more microorganisms into the mash. During the 63°C conversion step, approximately 0.1% of the vegetative cells and all bacterial spores will survive. Therefore, lowering of the pH during the first day of fermentation becomes

very important to control spore germination and vegetative growth. Mesophilic and thermophilic lactic acid bacteria will normally be in low numbers after conversion, but they are not greatly affected by low pH.

Equipment. Stainless steel and copper pipes are an integral part of a mash distribution system. Some gelatinized starch will adhere to the inside walls of the pipes, and its medium provides an excellent site for contaminating bacteria to grow. Occasionally, some of the gelatinized material will slough off, and large numbers of acclimatized bacteria will enter the fermentation. Sampling ports and elbows in the mash distribution system also are potential sites for bacterial build-up. In view of the foregoing, equipment design and process sanitation are important considerations in controlling the magnitude and physiological state of contamination microorganisms entering a fermentation.

Fermentation. The type of microorganisms which predominate are predicated upon the kind of fermentation. For example, the bacterial population in some molasses fermentations will consist almost entirely of *Leuconostoc* species. Those microorganisms present in a grain fermentation originate primarily from conversion malt and they include the coliform bacteria and members of the genera *Bacillus* (aerobic sporeformers), *Clostridium* (anaerobic sporeformers), *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Pediococcus*, *Leuconostoc*, and *Lactobacillus*.

During the course of a normal grain fermentation, most of the aerobic bacteria will increase in numbers for the first 18 to 22 h and then die off rather rapidly owing to the acidity produced by the lactic acid bacteria which grow and produce acid throughout the fermentation. Since the survival time of the fermentation. Since the survival time of the aerobic bacteria is short, they will have little to no flavor impact on the distillate. The clostridial bacteria usually do not pose a quality problem, because the ecological conditions of a fermentation are not favorable for their growth.

As the fermentation progresses, the lactic acid bacterial population will increase to several billion per mL. It is this group of microorganisms which have the greatest potential to influence the fermentation. If the lactic acid bacterial population is too high early in the fermentation, alcohol yield will be adversely affected. With respect to quality, these bacteria can be classified into three general

categories: (1) those that are beneficial, (2) those that are detrimental, and (3) those that grow but do not affect flavor or yield.

Aside from regulating distillate quality by preventing the growth of "off-flavor" bacteria, certain types of lactic acid bacteria are associated with changes in the congener profile of a distillate. Excessive amounts of yeast end products such as fusel oil, aldehydes, and diacetyl can be eliminated or greatly reduced by beneficial lactic acid bacteria. Desirable characteristics such as phenols in Scotch distillates, esters and acetates in Bourbon and rye distillates are related to the associative effect of the lactic acid bacteria and yeast.

Some malts contain a bacterial population which produces, very little lactic acid. In such instances, contaminating anaerobic and aerobic bacteria may grow throughout the course of fermentation and cause distillate quality defects. Clostridial bacteria produce objectionable solvent-types and butyric odors, and aerobic bacteria produce acetoin and acrolein. Since there are no controls with respect to bacterial types present on distiller's malt, selected strains of lactic acid bacteria are therefore useful for regulating quality uniformity. Certain strains have merit in correcting specific quality defects sometimes associating with rye fermentations.

14

Fermented Dairy Products

Introduction

The fermentation of milk with lactic acid bacteria is one of the oldest methods of food processing and food preservation still used by mankind. Although the art of preparing fermented dairy products has been practiced for ages, recent scientific and technological advances in starter culture management and process control have yielded a wide variety of products with improved chemical, physical nutritional and sanitary qualities.

Nearly every civilization has consumed fermented milk products of one type or another and these products have been, and still are, of extreme importance in human nutrition throughout the world.

Lactic Starter Cultures

Milk is an ideal culture medium containing many factors required for the growth of the fastidious lactic acid bacteria. Thousands of years ago, milk set aside for later use was fermented spontaneously by the natural milk flora. At some time it was recognized that a small portion of soured milk could be added to fresh milk (back-slop) to prepare additional sour milk.

Physiology and Biochemistry of Starters

The major lactic starter organisms and their physiological characteristics are shown in Table 14.1. Generally, *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus acidophilus* are used for acid production, *Streptococcus diacetylactis* and *Lactonacillus bulgaricus* for acid and flavor production and *Leuconostoc cremoris* and *Leuconostoc dextranicum* for flavor production. The combination of different species and strains

of these cultures allows a high degree of versatility in acid and flavor production.

Table 14.1 : Characteristics of Starter Organisms Used in Manufacturing Fermented Dairy Products

Culture	Incubation temperature	Growth 10°C	Growth 45°C	Acid production (% in milk)	Diacetyl production	Litmus reduction	Salt tolerance (% max)
<i>Streptococcus cremoris</i>	22-30	+	-	0.8-1.0	-	++	4.0
<i>Streptococcus lactis</i>	21-30	+	-	0.8-1.0	-	++	4.0-6.5
<i>Streptococcus thermophilus</i>	40-45	-	+	0.8-1.0	±	+	2.0
<i>Streptococcus diacetylactis</i>	22-28	+	-	0.8-1.8	+	++	4.0-6.5
<i>Lactobacillus acidophilus</i>	37	-	+	0.3-2.0	-	±	6.5
<i>Lactobacillus bulgaricus</i>	42	-	+	1.5-4.0	-	++	2.0
<i>Leuconostoc cremoris</i>	20-25	+	-	0.1-0.3	+	±	6.5
<i>Leuconostoc dextranicum</i>	20-25	+	-	0.1-0.3	+	±	4.0-6.5

The effect of starter culture compatibility on acid production and flavor in yogurt is illustrated in Table 14.2. Various commercial strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were combined in a 1:1 ratio and incubated at either 30°C (long incubation) or 42°C (short incubation) until the pH reached 4.2. The times required to reach the final pH and flavor score data for four representative starters

Table 14.2 Performance of Four Different Yogurt Starters Incubated at 30°C and 42°C

Parameter	Yogurt starter			
	1	2	3	4
<i>Incubation at 30°C</i>				
Time (h)	13	19	20	25
pH	4.0	4.1	4.1	4.2
Flavor score	3.6	2.3	2.0	2.0
<i>Incubation at 42°C</i>				
Time (h)	3.5	6.5	7.0	5.5
pH	4.2	4.2	4.2	4.2
Flavor score	4.0	2.7	3.0	3.0

are shown. Starter 1 produced acid rapidly at 30°C and 42°C, reaching the final pH within the expected time limit. Starters 2,3 and

4 were slow starters at both temperatures. Starter 1 produced a yogurt with an acceptable flavor (score of 3.0 or above) when incubated at either 30°C or 42°C. Starters 3 and 4 produced an acceptable yogurt only at 4.2°C. Starter 2 did not produce an acceptable yogurt at either 30°C or 42°C.

The basic biochemical reactions of lactic starter cultures in milk are illustrated in Figure 14.1. Homofermentative streptococci and lactobacilli split lactose to glucose and galactose. The glucose is then converted to pyruvate and then to lactic acid with trace amounts of acetic acid and carbon dioxide. Certain species and strains of homofermentative organisms may produce aroma and flavo-enhancing intermediates in addition to lactic acid. Moreover, under highly aerobic conditions, the homofermentative lactobacilli will produce acetic acid rather than lactic acid.

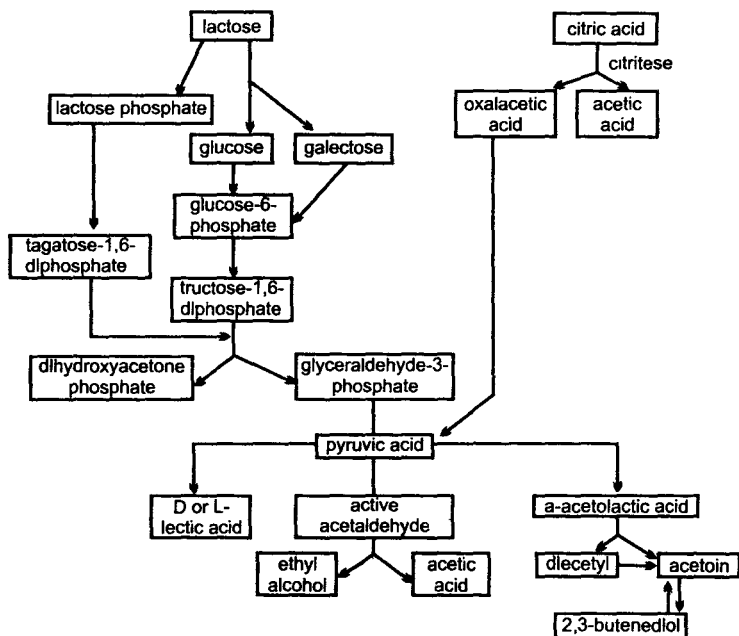


Fig. 14.1 : Biochemical reactions of dairy starter cultures.

The citric acid fermenting starters, *Streptococcus diacetylactis*, *Leuconostoc cremoris* and *Leuconostoc dextranicum*, convert citric acid to pyruvate and then to acetoin, diacetyl and carbon dioxide. Diacetyl imparts the characteristic 'buttery' flavor and aroma to

buttermilk and sour cream. Diacetyl has been thought to be formed by condensation of active aldehyde and acetylCoA or formed *via* the intermediated α -acetolactic acid. Under appropriate conditions, acetoin and diacetyl may be reduced to the flavorless and odorless compound, 2,3- butanediol (Figure 14.1).

Acetaldehyde, the major flavor component of yogurt, is produced in significant amounts through the symbiotic action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *Streptococcus diacetylactis*, a common buttermilk and sour cream starter, will produce acetaldehyde as well as acetoin. While the accumulation of acetaldehyde is important in yogurt flavor, it will result in undesirable green, harsh or yogurt flavors in buttermilk. These defects may be controlled by the inclusion of *Leuconostoc cremoris* in the starter culture, since this organism can convert the excess acetaldehyde to ethanol.

The chemical compounds produced during incubation of a commercial buttermilk starter are shown in Table 14.3. During fermentation only a small portion of the lactose is converted to lactic acid, while nearly all of the citric acid is utilized. However, the flavor compounds (acetoin, diacetyl and volatile acids) do not accumulate until after the pH drops to 5.0 . If the incubation period is extended beyond 16 hours, there will be loss in flavor due to the conversion of acetoin and diacetyl to 2,3-butanediol. Diacetyl production may be enhanced by adding 0.15% citric acid to milk prior to inoculation, monitoring the incubation time to allow sufficient acid production and synthesis of flavor compounds, aerating gently, chilling rapidly to avoid reduction to acetoin and 2,3-butanediol and by selecting starter strains which produce diacetyl in preference to acetoin.

Propagation and Management of Starters

Before the widespread availability and acceptance of commercial starter culture concentrates, cultures were maintained, propagated and evaluated at the plant level. This required specially trained personnel, separate culture handling facilities, extensive culture collections and time-consuming successive transfers to ensure the availability of sufficient active culture for inoculation. Today, standardized culture concentrates are purchased on a regular basis from commercial suppliers in three major forms: cryogenically frozen preparations designed for direct inoculation

into product mixes (direct sets), cryogenic concentrates for preparation of bulk starter (bulk sets) and lyophilized powders.

Table 14.3 : Compounds Produced in Milk Inoculated with a Mixed Starter of Lactic Acid Fermenting and Citric Acid Fermenting Organisms*

<i>Incubation time (h)</i>	<i>Titrateable acidity (%)</i>	<i>pH</i>	<i>Lactose (%)</i>	<i>Citric acid (%)</i>	<i>Acetoin (p.p.m.)</i>	<i>Diacetyl (p.p.m.)</i>	<i>Volatile acid (%)</i>
0	0.18	6.48	5.1	0.15	1.2	0.1	0.16
4	0.20	6.29	5.1	0.14	1.8	0.1	0.20
8	0.37	5.32	5.0	0.12	3.6	0.4	0.59
12	0.71	4.80	4.8	0.08	32.5	1.3	1.25
16	0.90	4.46	4.4	0.03	99.0	2.7	2.82

Propagation techniques for commercial bulk set cultures and lyophilized powders for use in buttermilk, cream cheese, cultured cream and cottage cheese are illustrated in Figure 14.2. Media are formulated at 10-12% solids using antibiotic-free skim milk, special phage inhibitory media or a combination of both. The media are then heat treated to destroy microbial contaminants and to stimulate the growth of starter cultures through partial hydrolysis of casein, release of sulfhydryl groups and conversion of lactose to formate. Some suggest that media for mother cultures be held at 90°C for 1 h or autoclaved at 120°C for 15-20 min, while media for intermediate and bulk cultures be held at 85-96 °C for 1 h. Autoclaving is not used for intermediate or bulk cultures since undesirable caramelized color and flavor may carry over into the final product.

Lyophilized powders may be shipped and stored for short periods of time without refrigeration. One package of dried culture may be used for the preparation of 750 ml of mother culture or up to 37.81 (10 gal) of intermediate or bulk culture, without preparation of mother culture. The performance of lyophilized powders may be limited, however, if they are used directly without one or two intermediated propagations.

Generally, the mixed strain starter cultures used for buttermilk, sour cream and cottage cheese are incubated at 20 to 21°C to maintain a proper balance between lactic streptococci and citric acid fermenting organisms. In cases where rapid acid production is desired, the incubation temperature may be increased to approximately 30°C.

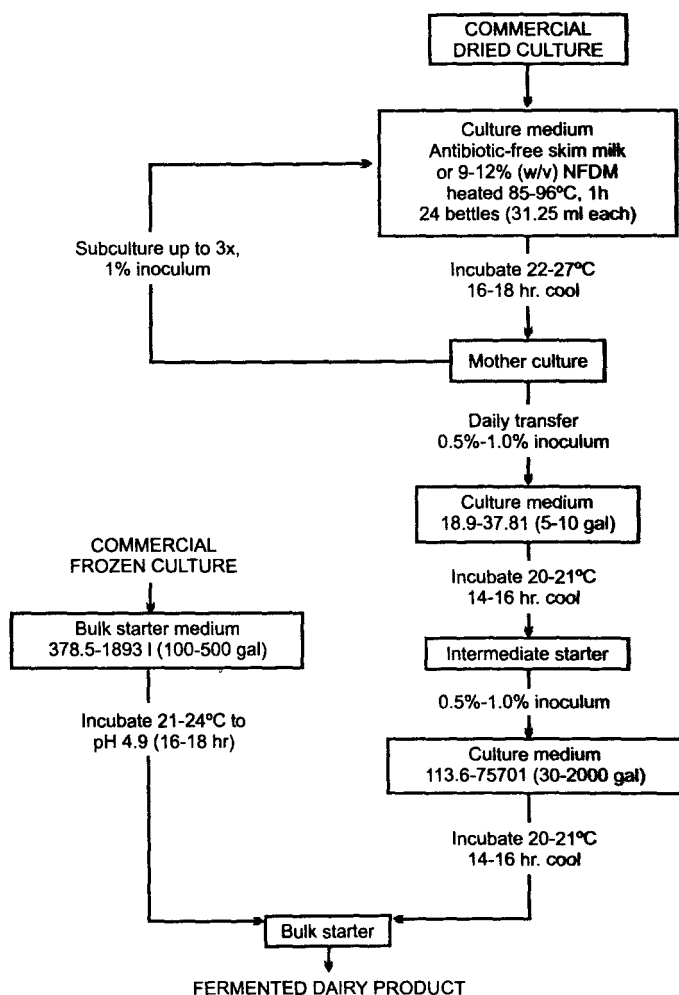


Fig. 14.2 : Preparation of bulk cultures for use in buttermilk, cultured cream and cottage cheese.

To assure maximum culture activity, media should be at the proper temperature prior to inoculation, inoculated media should not be agitated or aerated and cultures should not be held at incubation temperature for extended periods of time after coagulation.

By increasing the incubation temperature to 40–45°C, the procedures outlined in Figure 14.2 may also be used to prepare yogurt starters. Yogurt starter contains *Streptococcus thermophilus* and *Lactobacillus bulgaricus* growing in a symbiotic relationship. Incubation at 40 °C for a shorter time period will favor the growth of *Streptococcus thermophilus*, while incubation at 50°C for a longer time will favor the growth of *Lactobacillus bulgaricus*. The level of inoculum, incubation temperature and incubation time may be varied in order to maintain the two organisms in the desired 1:1 ratio during successive transfers.

Control of Starter Culture Inhibition

Inhibition of dairy starter culture growth may be noted by a decrease in the production of lactic acid (slow starter), by a complete cessation in the production of acid (dead starter) or by a decrease in the production of flavor compounds from citric acid. The primary causes of starter culture inhibition and suggested methods of control are summarized in Table 14.4.

Table 14.4 : Common Inhibitors of Dairy Starter Cultures

Substance	Suggested controls
Bacteriophage	Improve plant sanitation Sterilize with chlorine compounds Use multiple strain cultures, culture rotation and/or phage inhibitory media
Milk factors	
Antibiotics	Test milk supply and reject contaminated milk
Cleaning compounds	Monitor sanitation practices
Composition	Standardize composition Pasteurize to destroy natural inhibitors
Mastitis	Screen for mastitis
Rancidity	Avoid agitation of raw milk Pasteurize before homogenization

Bacteriophage infection

Bacteriophage infection is the major cause fo slow acid production by the lactic cultures. The bacteriophage adsorbs to the surface of sensitive host cells, penetrates the host with DNA, replicates and assembles the replicated phage particles, lyses the cells and releases up to 200 new phage particles which can attack additional phage-sensitive organisms. Since phage particles require

living cells to multiply, any material in which lactic cultures have grown becomes a potential source of phage contamination. Proper disposal of cottage cheese whey and careful cleaning and sanitation of equipment and rooms with chlorine compounds (200-300 p.p.m. solution for equipment and 500-1000 p.p.m. fog for rooms) are important for controlling bacteriophage infection.

If cultures are propagated in the plant, a separate culture preparation room must be provided to avoid contact with phage-containing materials. It is especially important to avoid contamination of the mother culture to ensure that phage multiplication does not occur during subsequent transfers to intermediate and bulk cultures. The use of specialized commercial media, which inhibit phage growth, reduces phage problems during culture propagation. These media are highly buffered and contain demineralized whey, non-fat dry milk, phosphate, citrate and yeast extract. The phosphate binds calcium and prevents calcium-dependent phage growth. Citrate contributes to buffering capacity and provides substrate for diacetyl production. The yeast extract provides growth factors for the lactic culture. Alternatively, in-plant propagation problems may be avoided altogether by using direct set cultures.

The final means of phage control used in the United States is the use of commercial multi-strain cultures which are rotated regularly on the basis of their phage sensitivity. The combination of good sanitation, phage inhibitory media and proper rotation of multi-strain cultures will reduce the probability of bacteriophage problems.

Milk factors

Pharmaceutical antibiotics may be present in the milk supply if milk is not withheld from the market for at least 96 h after treatment of cows for mastitis or other diseases. As shown in Table 14.5, as little as 0.05 IU of penicillin or 0.03 μ g of aureomycin will completely inhibit the growth of lactic starter cultures. Thus, all milk samples should be tested for antibiotics before use.

Many sanitizers used routinely on the farm and in the plant may be present as milk contaminants. 20-200 p.p.m. of chlorine compounds will inhibit acid production by most lactic starters, although individual strains vary in their sensitivity. Similarly, 50-

100 p.p.m. iodophores or 25-75 p.p.m. quaternary ammonium compounds will reduce or completely inhibit acid production. In addition, as little as 0.5 p.p.m. quaternary ammonium compounds will inhibit flavor production by the citric acid fermenting cultures.

Milk from mastitis-infected cows contains less lactose, less unhydrolyzed protein, more chloride and a higher pH than normal milk and, therefore, is considered to be a poor microbial growth substrate. Consequently, when such milk is used for the manufacture of cultured products, it does not support adequate acid development. The high concentrations of leukocytes associated with mastitis may also inhibit starter growth by phagocytic action, although this can be controlled by adequate heat treatment of the milk.

Table 14.5 : Minimum Levels of Antibiotic Required to Inhibit Growth of Dairy Starter Cultures.

<i>Starter culture</i>	<i>Penicillin (IU ml⁻¹)</i>	<i>Aureomycin (μg ml⁻¹)</i>
<i>Streptococcus lactis</i> 9	0.05	0.05
<i>Streptococcus thermophilus</i> H	0.05	0.03
<i>Lactobacillus bulgaricus</i> 444	0.10	5.0
<i>Lactobacillus bulgaricus</i> 488	0.10	3.0
<i>Lactobacillus bulgaricus</i> V12	0.05	0.3

Rancid milk results from the release of fatty acids from milk lipids through the action of inherent milk lipases or through the growth of lipolytic contaminants such as psychrotrophs. The presence of short chain fatty acids not only gives an objectionable flavor to the milk, but may also inhibit the growth of lactic starter cultures. Although both milk and microbial lipases are destroyed by pasteurization, problems may occur in raw milk which has undergone vigorous agitation or homogenization, temperature activation of lipase or extended storage.

General Manufacturing Principles

Fermented dairy products can be divided into three classes: liquid products which include acidophilus milk, buttermilk, kefir and koumiss; semi-solid products which include cultured cream and yogurt; and the unripened soft cheeses which include bakers' cheese, cottage cheese, cream cheese and quarg (Table 14.6). Buttermilk, cultured cream and cream cheese are prepared from

starters containing lactic streptococci (*Streptococcus lactis* and *Streptococcus cremoris*) and citric acid fermenting organism (s) (*Streptococcus diacetylactis* and/or *Leuconostoc cremoris*). Bakers cheese, cottage cheese and quarg utilize only the lactic streptococci. Lactobacilli are used for the preparation of acidophilus milk (*Lactobacillus acidophilus*), kefir (*Lactobacillus caucasicus* plus lactic streptococci, *Leuconostoc* spp, and yeasts), koumiss (*Lactobacillus bulgaricus* plus *Torula* yeast) and yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*).

Processing Steps and Ingredients

Scientists described the general design of plants used for the manufacture of cultured dairy products. Each plant is equipped with a separate receiving room to receive, weigh and store milk and other raw materials. The plant also contains an ambient-temperature dry storage area, a refrigerated storage area, a processing room, a packaging room, a utility room, a culture propagation room and a quality control laboratory. Dry storage is used for heat-stable raw materials and packaging supplies, while the refrigerated storage area is used for fruits, other heat-labile raw materials and finished products. The processing room contains the equipment and controls for the separation, standardization, pasteurization and homogenization of milk and the formulation, inoculation and incubation of product. Plants for the manufacture of cottage cheese contain cheese vats with mechanical agitation devices, cutting knives, and systems for whey draining, curd washing and creaming. The utility room is used for plant maintenance and engineering services. A separate culture preparation room is required only if cultures are maintained and propagated in the plant. Finally, a quality control program is needed to monitor culture preparation, the quality of raw materials, process control, product composition and shelf life to ensure compliance with regulatory and company standards.

We discussed the importance of environmental sanitation in the production, processing and distribution of milk and milk products. These authors define environmental sanitation as the 'maintenance of all substances, surfaces and materials which contact the product directly or indirectly in a state which (a) is free from disease-producing bacteria and toxic-substances, (b) is free from foreign materials, (c) has low bacterial count, (d) does not influence product

Table 14.6 : Major Fermented Dairy Products Other Than Hard Cheeses

<i>Product</i>	<i>Process</i>	<i>Starter cultures</i>
Liquid products		
Acidophilus milk	37-40°C, 16-18 h	<i>Lactobacillus acidophilus</i>
Buttermilk	22°C, 18 h	<i>Streptococcus cremoris</i> or <i>S. lactis</i> , <i>S. diacetylactis</i> or <i>Leuconostoc cremoris</i>
Kefir	18-22°C, 12h or 10°C, 1-3d	Kefir grains (<i>Streptococci</i> <i>spp.</i> , <i>Lactobacillus caucasicus</i> , <i>leuconostoc spp.</i> , Yeasts
Koumiss	28°C, 18h	<i>Lactobacillus bulgaricus</i> <i>Torulopsis holmii</i>
Semi-solid products		
Cultured cream	22°C, 18h	<i>Streptococcus cremoris</i> or <i>S. lactis</i> , <i>S. diacetylactis</i> or <i>Leuconostoc cremoris</i>
Yogurt	43-45°C, 3h	<i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i>
Unripened soft, cheeses		
Bakers' cheese	31°C, 5h	<i>Streptococcus cremoris</i> or <i>S. lactis</i>
Cottage cheese	22°C, 18 h or 35°C, 5h	<i>Streptococcus cremoris</i> <i>S. lactis</i>
Cream cheese	22°C, 18 h	<i>Streptococcus cremoris</i> or <i>S. lactis</i> , <i>S. diacetylactis</i> or <i>Leuconostoc cremoris</i>
Quarg	31°C, 5h	<i>Streptococcus cremoris</i> or <i>S. lactos</i>

safety or keeping quality and (e) promotes the high nutritive value of the product'. To achieve this, some consideration be given first to the layout of rooms and the ventilation system to exclude outside contamination, as well as to prevent cross contamination between raw materials and finished product within the plant. Secondly the floors, walls and ceilings should be constructed of smooth, impervious materials. Floors must be sloped to ensure rapid drainage of water, whey and other liquid waste. Thirdly, the design and installation of equipment and pipelines and the materials used in their construction are of critical importance since the product is in direct contact with their surfaces during the entire production

process. Stainless steel and glass are used routinely for equipment and pipelines since these materials provide surfaces which are smooth, impervious, corrosion-resistant, non-toxic and easily cleaned. Fourthly, all surfaces which come in contact with the product must be cleaned immediately after use to remove all evidence of soil, and then should be sanitized with a bactericidal agent. Other plant surfaces such as the floors, the walls and the ceilings should be given less rigorous, though similar, attention.

The general process steps used to manufacture fermented dairy products are outlined in (Table 14.7). While milk from cow, water buffalo, goat mare, sheep and sow are used for cultured products in many parts of the world. All milk must be of high quality and free from inhibitors to ensure optimum culture growth. After reception at the plant, milk may be stored for up to 72 h at 10°C in large vertical silos holding up to 100,000 l. Extraneous matter (including leukocytes, some bacteria, dust and dirt) is removed from the milk in a high speed centrifuge known as a clarifier. Milk is then centrifuged using a separator or standardizing clarifier to separate the cream and skim milk for standardization of fat levels.

Raw whole milk (3.7% butterfat) may be separated into full cream (37% fat) and skim milk (0.1% fat) (Figure 14.4). By adjusting the cream flow valve, the relative quantity of butterfat can be standardized to that of low fat milk (2% fat), light cream (18% fat) or other levels. Non-fat dry milk (NFD) is prepared from condensed skim milk which has undergone either spray or roller drying. These ingredients are blended together in a mix tank at 50°C at a predetermined level of fat and solids-non-fat (Table 14.7).

Milk is pasteurized by vat, HTST or UHT processes. Vat pasteurization is conducted at 63°C (66°C if sweeteners are present in the mix) for a minimum of 30 min. For HTST, the equivalent temperature is 73°C (75°C with sweeteners) for 15 s. UHT systems are used at 90-148°C for 2 s. Generally, standardized mixes are heated to above pasteurization temperatures in order to destroy contaminating microorganisms, to release growth factors, to generate microaerophilic conditions required for starter culture growth and to achieve the proper body and texture in the fermented product. One exception is cottage cheese manufacture, where skim milk is only heated at 72°C for 16-17s.

Table 14.7 : Process Steps for the Manufacture of Fermented Dairy Products

<i>Process step</i>	<i>Remarks</i>
1. Procurement of milk from the farm	Antibiotic-free milk from healthy cows should be cooled in refrigerated bulk tanks to 10°C in 1 h and below 5 °C in 2 h to ensure microbiological control. Milk pick up should be at 48 h intervals in insulated tanks. Avoid unnecessary agitation to prevent lipolytic rancidity problems in milk
2. Reception and storage of milk in plant	Quality of milk checked prior to reception and storage at 10°C for not more than 72 h
3. Centrifugal clarification and separation	Leucocytes and sediment are removed. Milk separated into cream and skim milk, or standardized to a specific fat level
4. Mix preparation	Ingredients are blended together to a desired formulation at 50°C in a mix tank
5. Heat treatment	Mix is heated above pasteurization (85-95°C, 10-40min). Heat treatment destroys contaminating microorganisms, releases growth factors, generates micro-acrophilic conditions and improves body and texture in fermented product
6. Homogenization	Mix is homogenized in two stage process to prevent aggregation of fat globules during incubation and storage of products. Aids in uniform dispersion of stabilizer and other mix components
7. Inoculation and incubation	The mix is cooled to incubation temperature, inoculated with 0.5-5% starter culture and incubated quiescently until desired pH level is reached
8. Cooling, incorporation of fruit and flavoring and packaging	The coagulated mix is cooled to 45-22°C, depending on the product, Fruit, fruit-flavors, other flavoring or seasoning ingredients are incorporated before packaging
9. Storage and distribution	Low temperature storage ensures adequate shelf life by decreasing the rate of physical, chemical and microbiological degradation

After heating, the mix is homogenized in a two-stage treatment. The first stage (6.8-13.7 k Pa) reduces the average size of the milk fat globules from a 4 μm diameter to 1 μm . The second stage (3.4 k Pa) breaks apart fat globule clusters to prevent creaming. Stabilizer may also be added at this time to improve the body and texture of the product.

The mix is cooled and transferred to a jacketed, culturing vat equipped with an agitation system. The mix is inoculated either with a vat set culture concentrate or with a bulk starter, and is incubated until the desired pH is achieved, Rennet may also be added to increase the body of the product. The fermented mix is then cooled quickly to 5-22°C, depending on type of product to be manufactured. High quality fruits, fruit flavors and other seasoning and flavoring ingredients are incorporated prior to packaging. Proper storage of the product at low temperature will ensure an adequate shelf life by decreasing the rate of physical, chemical and microbiological degradation.

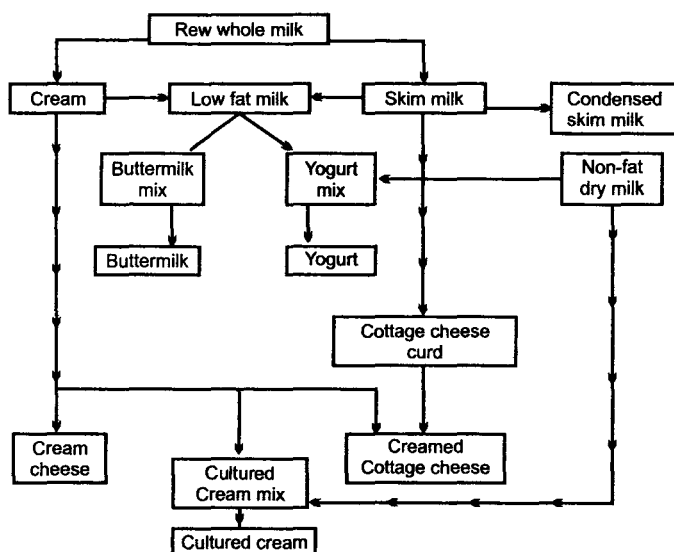


Fig. 14.3 : Milk ingredients used for the manufacture of fermented dairy products.

The manufacture of cottage cheese curd requires several modifications to the general scheme. Since skim milk is used rather

than a mix, cottage cheese requires a less severe heat treatment and no homogenization. After inoculation and coagulation, the curd is cut, cooked, drained and rinsed. A special cream 'dressing' (cream, salt and stabilizer mixture) is then mixed with the dry curd before packaging and storage.

Use of Direct Acidification

Direct acidification has been as an alternative to fermentation in the production of sour milk and imitation cultured milk products. Commercial processes for buttermilk and cottage cheese have also been introduced.

The base for these products consists of dairy ingredients or an emulsified vegetable oil stabilized with sodium caseinate. Food-grade acidulant replaces the starter culture used in fermented products. Citric, phosphoric, gluconic and lactic acids are used, although the high cost of lactic acid limits its application. Alternatively, gluconic acid may be formed in milk by the hydrolysis of glucono- δ -lactone. Culture distillates containing diacetyl are added for flavor. The body and texture of the fermented product are simulated by the addition of emulsifiers and stabilizers including vegetable gums, starch, carrageenan, gelatin, partial glycerides, caseinates and sodium phosphate. Specific processing steps used for the manufacture of directly acidified sour cream and cottage cheese will be given later.

Manufacture of Liquid Products

Acidophilus and Sweet Acidophilus Milks

Certain strains of *Lactobacillus acidophilus*, a normal inhabitant of the human intestine, can be transplanted to the intestine and provide therapeutic benefits by suppressing the growth of undesirable microorganisms.

The general preparation of acidophilus milk has been discussed. Low fat or skim milk should be sterilized at 120 °C for 20 min (15 p.s.i.), cooled to 38°C, inoculated with 5% *Lactobacillus acidophilus* and incubated for 18 to 24 h until a curd forms with 1% acidity. In contrast, some recommended ultraheating whole or skim milk at 98°C for 30 min or 145°C for 2 to 3 s rather than autoclaving to increase the growth rate of the organism. He also noted the

culture should be incubated at 37°C only until the pH reached 4.7(0.65% acidity), since increased acid production decreases cell viability. While fresh acidophilus milk contains more than 500 million viable cells per ml, viability decreases rapidly during storage. In addition, the strong acid flavor of this product has little appeal in the United States.

A sweet acidophilus milk, in which *Lactobacillus acidophilus* cell concentrates are added to fresh low fat milk, has been marketed to overcome the problems of culture viability and consumer appeal. Sweet acidophilus milk contains more than 2 million viable cells per ml, yet retains its sweet, unfermented flavor if held at refrigeration temperatures during distribution and storage.

Cultured Buttermilk

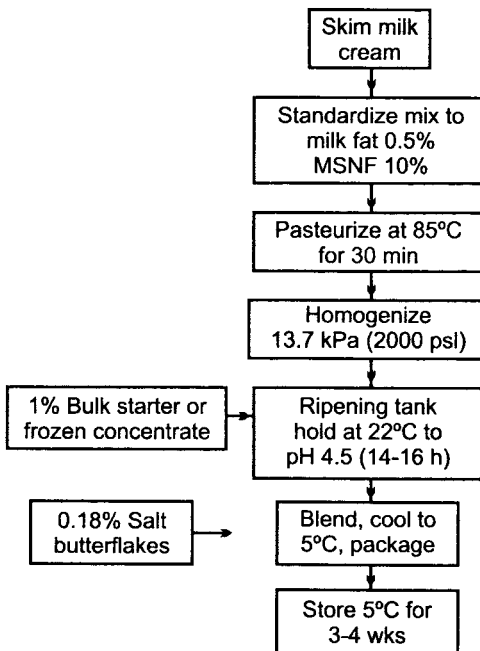


Fig. 14.4 : Flowchart for the manufacture of cultured buttermilk
(From Chandan and Shahani, 1982).

Cultured buttermilk is prepared from skim or low fat milk fermented with *Streptococcus lactis* or *Streptococcus cremoris* for acid production and *Leuconostoc cremoris*, *Leuconostoe dextranicum* or *Streptococcus diacetylactis* for flavor production (Figure 14.4). Although milk fat levels from 0.5–1.8% have been used, the proposed federal buttermilk standards require a maximum fat content of 0.5% and a minimum milk solids-non-fat (MSNF) of 8.25% (Anon, 1977). The product must also possess a minimum titratable acidity of 0.5% calculated as lactic acid. In addition to traditional dairy ingredients, any milk-derived ingredient may be used to standardize the solids content of the mix provided there is no decrease in either the ratio of protein to MSNF or the protein efficiency ratio. Other legal ingredients include characteristic flavorings (starter distillate, butter flavor, diacetyl), nutritive carbohydrate sweeteners, stabilizers and colorings. Vitamin fortification with a minimum of 2000 IU of vitamin A and 400IU of vitamin D per 0.95 l is also allowed. Milk which is low in citric acid is normally supplemented with 0.20–0.25% sodium citrate for flavor enhancement. In certain parts of the United States, butter granules or flakes are added to the finished buttermilk to give a heavier product with smoother taste. Butterflakes are prepared by churning 18–20% fat cream, or by spraying melted butter oil at 71.1°C onto the chilled buttermilk.

In general, a good buttermilk flavor and texture will be produced by using high quality milk ingredients formulated at the proper solids level, heating at 85°C for 30 min. using active starter, incubating at 22°C to maintain the balance between the starter organisms, breaking the curd at appropriate acid level, cooling rapidly with gentle agitation to avoid destruction of diacetyl and avoiding contamination problems through proper sanitation. The curd should be broken at pH 4.65–4.7 which is equivalent to a titratable acidity (TA) of 0.85% for mix containing 9% MSNF and a TA of 0.90% for 10% MSNF or a TA of 0.95% for mix containing 11% MSNF.

The use of 'return milk' poor quality ingredients or the presence of microbial contaminants may cause bitter, stale, cheesy, yeasty, putrid or unclean flavors. Also, poor flavor development may result from low acidity and/or diacetyl production due to poor culture balance, poor starter activity or improper handling. On the other hand, excessive production of acid or diacetyl may cause harsh or

bitter flavors, although harshness may be cut by the addition of 0.1–0.2% salt. Acetaldehyde accumulation gives a green or yogurt flavor. This can be prevented by using cultures free of contamination with *Streptococcus diacetylactis*, or by using direct vat inoculation of each batch of buttermilk.

A weak bodied, thin buttermilk is produced if the mix contains less than 9% milk solids, improper heat treatment is used, the culture is inactive, acid development is low or there is excessive agitation. A thick, viscous product will occur if the mix contains high solids, excess acid, insufficient agitation, slime forming organisms or other microbial contamination. A grainy or lumpy buttermilk will occur if excess acid is produced, the ingredients are not dissolved completely, the heat treatment is too low, the incubation temperature is too high, there is insufficient agitation during breaking or poor quality milk is used.

Manufacture of Semi-Solid Products

Cultured Cream

Cultured cream or sour cream is prepared by fermenting a minimum of 18% fat cream to a titratable acidity of 0.5% using the same lactic starter as buttermilk. Optional ingredients include stabilizers, rennet, salt, nutritive sweeteners, flavorings and colors. Up to 0.1% sodium citrate may also be added for flavor enhancement.

The procedures for preparing cultured cream, long life cultured cream, sour cream dip and acidified sour cream are shown in Figure 14.5. Raw cream should be fresh and of high bacterial quality. Heavy, full-bodied products are obtained by formulating mixes with at least 6.8% MSNF, using HTST pasteurization (74°C for 16 s) rather than vat pasteurization, homogenizing in two stages at 72°C and 17.2 kpa, adding rennet, mixing without incorporation of air and allowing sufficient acid production.

Long-life or hot-pack cultured cream is prepared by adding 0.20–0.5% stabilizer to normal cultured cream and then heating, homogenizing and hot-packing the mixture into glass containers or metal cans. Heating destroys proteolytic enzymes of the lactic acid streptococci which can cause bitterness during storage. In addition, the combination of the heat treatment and natural vacuum which forms after cooling prevents the growth of molds and yeasts and minimizes oxidative deterioration of the fat. It was noted that hot-

pack cultured cream may retain its quality for as long as 4-12 months before oxidation occurs.

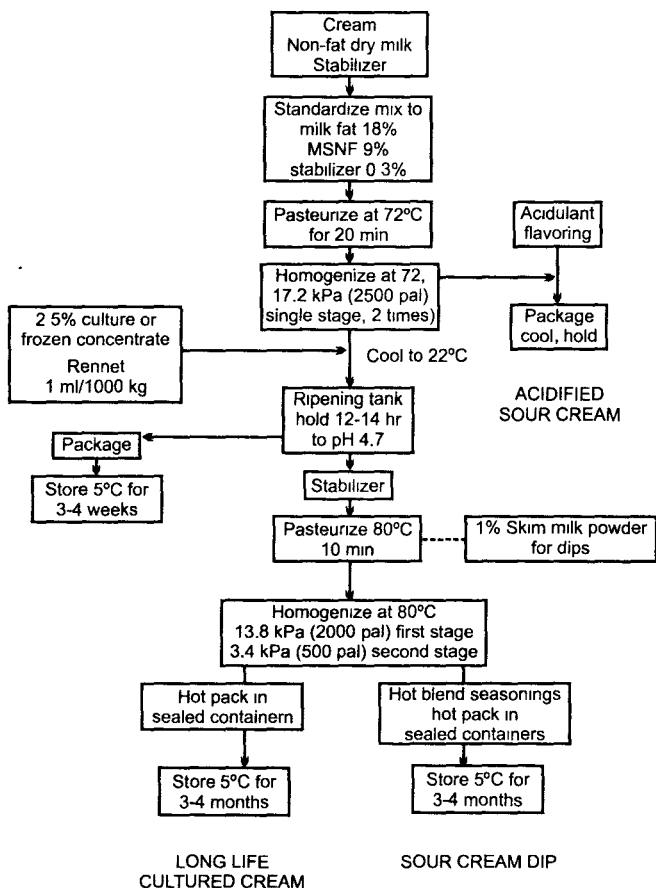


Fig. 14.5 : Flowchart for the manufacture of acidified sour cream, cultured creams and dips.

Sour cream dips are prepared by adding appropriate seasonings to cultured cream (16-18% fat) or long-life cultured cream. Cheese condiments are introduced at the rate of 20% for blue cheese and 100% for cheddar cheese; concentrated flavorings such as horseradish are added at 1.5%. To improve the body and stability of dips prepared by the hot-pack process, Chandan (1982) suggests the incorporation of 1-2% non-fat dry milk and 0.8-1.0% stabilizer

at 80°C prior to homogenization. If the viscosity of the product is too heavy after ingredient addition, a lower fat mix or a longer agitation time should be used.

Cultured half and half has an advantage over cultured cream in terms of lower price and lower caloric content, but has a lower viscosity which makes it subject to wheying off. It is manufactured using the same process as cultured cream except that the standardized mix contains 10.5-18% fat and 10-12% MSNF. Sour half and half has also been used as a base for low-calorie creamy salad dressing since it contains 10.5% fat versus the 30-80% fat in regular dressing.

Sour cream can also be prepared by direct acidification. Acidulant and flavoring are added to the homogenized mix at 22°C. The acidified product is then packaged, cooled to 4°C and held for 24 h before distribution. Acidified sour cream provides an excellent, low-cost cream base which is used in many food products. Federal standards require, however, that sour cream prepared by direct acidification be labeled as an 'acidified' product.

Yogurt

The proposed Federal Standards of Identity yogurt as containing 3.25% milk fat, low-fat yogurt as containing 0.5% to 2% milk fat and non-fat yogurt as containing less than 0.5%. As in buttermilk, the use of milk-derived ingredients is permitted provided the ratio of protein to MSNF and the protein efficiency ratio are not decreased. Additives include nutritive carbohydrate sweeteners, coloring, stabilizer, fruits and fruit flavors. The starter culture must contain *Lactobacillus bulgaricus* and *Sterptococcus thermophilus*.

According to a recent survey, plain yogurt remains the most popular variety with 18.3% of the sales. The most popular flavor is strawberry at 13.7% followed by raspberry and peach at 9.6% each. The next most popular flavors are blueberry, pineapple, lemon, cherry, apple, boysenberry and blackberry.

The basic outline for the preparation of four basic types of yogurt is given in Figure 14.6. The set-type plain yogurt and sundae-style fruited yogurt are packaged before incubation, while the Swiss-style fruited yogurt and stirred-type plain yogurt are cultured in vats before packaging. From 0.5-0.7% stabilizer is added to the latter types in order to impart gel structure, to ensure a smooth body and

texture and texture and to prevent wheying off or syneresis after packaging. Yogurt that is overstabilized will be solid ion consistency, while yogurt which is under-stabilized will be liquid.

Generally, yogurt mixes are formulated to 10-12% MSNF. Yogurt prepared from mixes containing 12-15% solids will have a medium firm, custard like consistency while yogurt from mixes containing more than 15% solids will have a firm, heavy body. The actual consistency is also dependent on the amount of stabilizer, if any, and the heat treatment of the mix.

Gelatin, seaweed gums (alginate, sodium alginate and carrageenan), vegetable gums (carboxymethyl cellulose, locust bean gum and carob gum) and pectin are used as stabilizers in yogurt. It noted that yogurt stabilizers should not impart flavor to the product, should be active at low pH and should be dispersible at the normal temperature of the yogurt. He also noted that 0.3-0.5% gelatin gives a smooth, shiny appearance to refrigerated yogurt, and a pudding-like consistency to frozen yogurt. The activity of gelatin is temperature dependent and will degrade at high temperatures. In contrast, the seaweed gums, guar gum and carboxymethylcellulose are heat-stable.

A heat treatment of 85°C for 30 min is needed (a) to destroy microbial contaminants, (b) to provide a microaerophilic environment and release peptone, sulfur groups and formate required for starter culture growth and (c) to denature and coagulate milk albumins and globulins and thus increase the viscosity and improve the body and texture of the final product. Temperatures above 90°C for over 30 min will decrease water binding capacity of the whey proteins, weaken the yogurt curd and increase syneresis in the product. Proper homogenization of the mixture improves the texture and decreases surface creaming and wheying off.

It was noted three methods of preparing fruited yogurts: the fruit-on-bottom or Eastern sundae-style yogurt, Western sundae-style yogurt and stirred or Swiss-style yogurt. In the Eastern sundae-style, 59 ml (2oz) of fruit preserves are layered in the bottom of the container prior to the addition of 177 ml (6oz) of unflavored, unsweetened yogurt mix which has been inoculated with starter culture. The containers are incubated until their contents reach pH 4.2-4.4 and then placed in refrigerated rooms for cooling. In

contrast, Western sundae style yogurt has fruit preserves or fruit preparations on the bottom and yogurt containing sweeteners, coloring and flavoring (2-4%) on the top. Identity standards require that fruit preserves contain 55% sugar and a minimum of 45% fruit, usually frozen fruits or juices, which are cooked to a final soluble solids content of 65%-68%. Fruit preserves also contain approximately 0.5% pectin and sufficient citric acid or other food grade acidulant to adjust the pH to 3.0-3.5%.

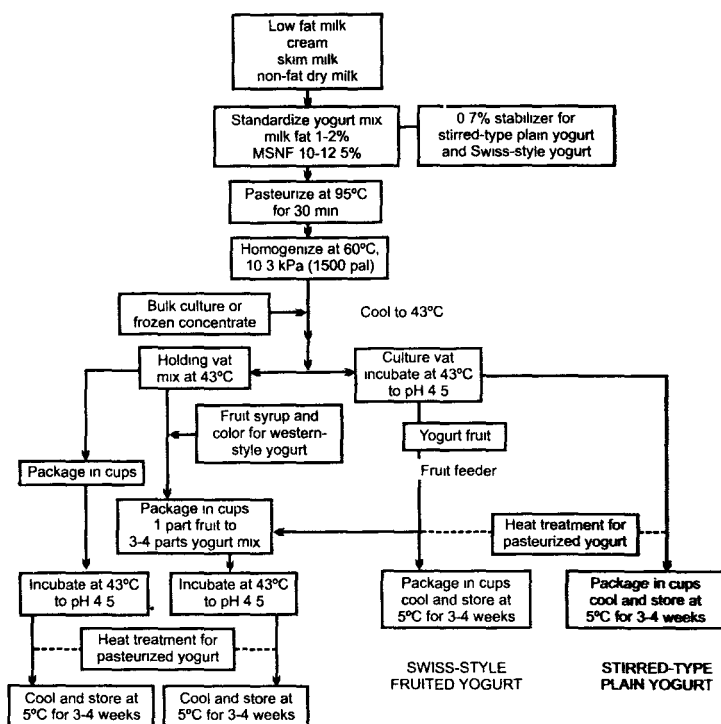


Fig. 14.6 : Flowchart for the manufacture of low-fat plain and fruited yogurts (From Chandan, 1982)

In Swiss-style yogurt the fruit is added after fermentation. To increase smoothness, the mixture may be pumped through a valve or stainless steel screen prior to packaging. The packaged mixture is stored under refrigeration for 48 h to allow for thickening. The special fruit bases used in stirred yogurt contain 17-41% fruit, 22-40% sugar, 10-24% corn syrup solids, 3.5-5.0% modified food starch,

0.1% natural or 1.25% artificial flavor, 0.01% color, 0.1% potassium sorbate and sufficient citric acid to adjust the pH to 3.7–4.2. In addition, calcium chloride and food-grade phosphates may be used in specialized preparations. Fruit bases contain 60–65% soluble solids, a standardized viscosity and a total microbial count of less than 500 cfu per gram with coliform, yeast and mold counts of less than 10 per gram.

We reported on the defects found in plain yogurt and Swiss-style and sundae-style strawberry yogurts. Flavor problems included: (a) unnatural flavoring due to poor quality fruit and/or flavoring materials; (b) low flavor and/or low acid development due to insufficient heat treatment of mix, poor culture activity, high incubation temperature, excessive sweetener or improper flavoring; (c) high acid flavor due to culture imbalance, increased incubation temperature and time, insufficient cooling or inadequate refrigeration during storage; (d) old ingredient flavor from aged, stalo or otherwise poor quality ingredients; (e) over-sweetened flavor due to excess sweetener, excess flavoring materials, insufficient acid production or insufficient flavor production; and (f) bitterness due to poor or contaminated cultures, poor quality dairy ingredients, poor quality fruit or flavoring, psychrotrophic contamination or excessive preservatives.

Body and texture defects included: (a) lumpy texture due to improper or excess stabilizer, uneven acid development, bacterial contamination, poor incorporation of fruit or inadequate cooling after packaging; (b) weak curd due to low solids and/or protein in mix, improper use of stabilizer, excessive heat treatment, high incubation temperature, insufficient acid, excessive agitation, excessive line and pump pressure or microbial contamination; (c) gel-like or firm curd due to high solids in mix, excess stabilizer or dehydration because of poor packaging materials; (d) grainy texture due to high acid milk, unstable casein, improper stabilizer selection, poor cultures or improper culture balance, high incubation temperature, fast acid development or homogenization at too high a temperature; and (e) ropy texture due to over pasteurization, improper choice of stabilizer, culture selection, microbial contamination or excessive sweetener.

While post-production pasteurization of yogurt at 60–65°C will increase the shelf life to 6–8 weeks at 12 °C it will also destroy the

starter culture and any benefits associated with the presence of a viable culture. For this reason the proposed federal standards will require any thermal processed yogurt to be labeled as 'heat treated after culturing'.

Several liquid products such as yogurt drink (3.5% butterfat, 8.25-10.0% MSNF), low fat yogurt drink (0.5-2.0% fat) and non-fat (less than 0.5% fat) yogurt drink have also been formulated to meet the proposed Standards. Stabilizer (0.2-0.3%) and 4.0% sucrose are also added. After pasteurization and homogenization, the product is incubated at 45°C for 8-9 h until the pH drops to 4.3. The mixture is cooled to 32°C with agitation. Sugar syrup and flavoring are added and the mixture is cooled to 4°C and packaged. The product is then held overnight at 4°C to allow development of body, texture and mouth feel. Carbonation has also been added to yogurt beverages.

Some described the manufacture of yogurt cheese. Pasteurized mix is inoculated with 2.5% yogurt starter at 35°C, incubated for one hour and then inoculated with a 1% commercial starter containing *Streptococcus lactis* or *streptococcus cremoris* along with rennet and annatto coloring. The milk is held for 2-4 h until the pH reaches 4.7. The curd may be cut, or may be dipped using the same procedure as bakers cheese. The final product resembles a cream cheese with a smooth grained texture and mild nutty flavor.

Manufacture of Unripened Soft Cheeses

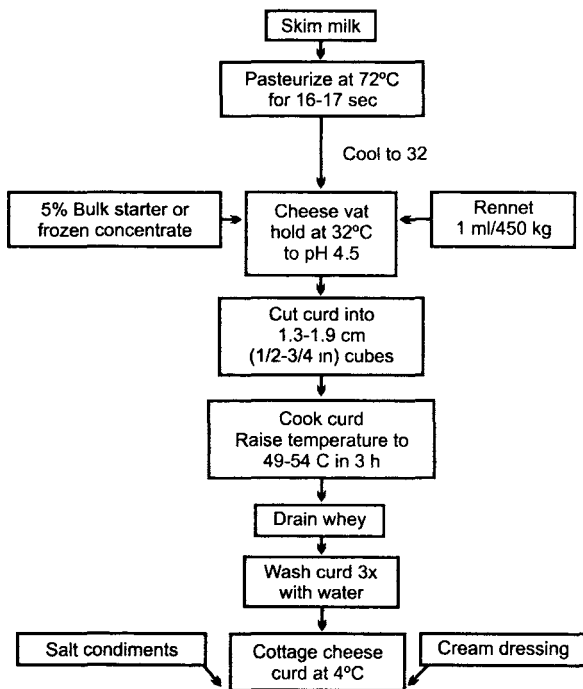
Cottage Cheese

Cottage cheese is prepared in the United States by a short set method which uses 5-7% starter and an incubation of 4.5-5.5 h at 31-32°C, by a medium set method which uses 2-4% starter and an incubation of 8-10h at 27-28°C and by a long set method which uses 0.1% starter and an incubation of 12-16 h at 21-22°C.

The short set method is illustrated in Figure 14.7. Raw skim milk containing at least 9% solids is pasteurized, cooled to 32°C and inoculated acidity of the mix is determined and the mix held for 1.5 h, stirring every 30 min. At this point the titratable acidity should be 0.05-0.07% greater than the initial reading. Some suggests that if acidity increase is less than 0.05%, an additional 1% of culture should be added for each 0.01% increment below 0.05%. Rennet is

added at the rate of 1 ml per 454 g for large and medium curd cottage cheese, and 0.3-0.5 ml per 454 g for small curd. The vat is covered and allowed to incubate for an additional 2h before the curd formation is checked.

Cottage cheese curd is ready to be cut when the titratable acidity of the whey is 0.34 to 0.36% higher than the initial value, when the curd pH is 4.6 -4.7 or when the end point of the acid coagulation test is reached. The curd is first cut lengthwise with a horizontal knife. It is then cut lengthwise with a vertical knife and finally cut crosswise with a vertical knife. The size of the curd varies from 0.95 cm for small curd to 1.9 cm for large curd cottage cheese.



CREAMED COTTAGE CHEESE

Fig. 14.7 : Flowchart for the manufacture of cottage cheese.

The curd is held without agitation for 10 min to allow the curd surfaces to firm. The curd is pushed to the center of the vat and cooked by raising the temperature to 49-54°C within 3 h. The curd

is then washed three times with water which has been charcoal-filtered to remove off-flavors, acidified with citric or phosphoric acid to pH 4.0-5.0 and chlorinated to kill spoilage organisms. After the final wash, the curd is trenched and allowed to drain for 30-60 min before creaming.

Cottage cheese dressing normally contains 12.5% fat, 8.5% MSNF, 2.7% salt and 0.25% stabilizer, while low fat dressing contains 3.0% fat, 15.0% MSNF, 2.7% salt and 0.25% stabilizer. Dry ingredients are mixed separately and added to a mixing vat by centrifugal pump. After mixing, the dressing is vat pasteurized at 75-77°C for 30 min, homogenized at 57°C and 13.8°C kPA and cooled to 4°C. One part dressing is then mixed with two parts dried cheese curd. Federal standards require creamed cottage cheese to contain not more than 80% moisture and not less than 4% fat *versus* 0.5-2.0% fat in low-fat cottage cheese. Other ingredients include silicone antifoams, sorbate preservatives and flavorings. In addition, citric acid fermenting organisms may be incorporated into the cream dressing to enhance the flavor and increase shelf life.

Bakers' Cheese

Bakers' cheese, cream cheese and quarg are all unripened soft cheeses, which differ from cottage cheese in the manner of whey separation, whey drainage, individual curd structure and cream incorporation. Bakers' cheese is utilized widely for the preparation of pastries, cheese cakes and cake decorating materials. Kosikowski noted bakers' cheese is prepared by adding 5% lactic starter and rennet to skim milk and then incubating the mixture at 31.1°C for approximately 5h until the pH reaches 4.4. The mixture is chilled, placed in mesh bags overnight for whey drainage, bulk packed and stored frozen. The product should have a dry, pliable, smooth curd which is similar in consistency to bread dough. High acid formation gives the cheese an undesirable harsh flavor, while low acid production gives the cheese a grainy texture and decreases its absorption of milk in baked products.

Cream Cheese

Federal Standards of Identity require that cream cheese contains at least 33% fat and not more than 55% moisture. To produce cheese of legal composition, the mix must contain a minimum of 11.5% fat and 7.8% MSNF. Standardized cream cheese

mix is pasteurized at 71.1°C for 30 min, homogenized at 17.2 kPa, cooled to 31.1°C and inoculated with starter culture (5%) and rennet (1 ml 454 kg⁻¹). The mixture is held for approximately 5 h until the pH reaches 4.6. The curd is agitated, heated to 54°C cooled to 32.2°C diluted with water to facilitate whey drainage and then further cooled to 7.2 °C . The whey is drained by mechanical centrifugation or by transferring to mesh bags.

For the cold pack process, dried curd is blended with 0.5% stabilizer and 1% salt before packaging and storage at 4.4°C . While this product possesses an excellent flavor and texture, it is subject to yeast or mold spoilage within 1 to 2 weeks. For the hot pack process, dried curd is mixed with 1.0% salt, heated to 73.9°C and then standardized for fat and total solids using a special mixture containing 15% SNF and 0.35% gum at pH 4.5. The standardized mix is pasturized by holding at 73.9°C for 30 min, homogenized and packaged hot. Hot pack cream cheese is free of coliform, yeasts and molds, but is susceptible to chemical deterioration after 2 to 3 months storage.

If desired, condiments such as pimentos, relish, chives, scallions, olives, cherries, pineapples or nuts can be added to the hot mixture immediately after homogenization. Since these additions reduce fat levels, the minimum legal fat requirement for cream cheese containing condiments is 27% rather than 33% fat.

Neufchatel cheese, containing from 20 to 33% fat and not more than 65% water, has the same flavor as cream cheese but a slightly more grainy texture. It is prepared from a standardized mix containing at least 5% fat and 8.4% SNF using the same procedures outlined for cream cheese.

Quarg

Quarg or quark is an unripened fresh cheese similar to bakers' cheese. Quarg may be made from skim milk, reconstituted skim milk powder or standardized mixes containing up to 12% added fat. Its smooth texture and mild acid flavor increase with increasing levels of fat. Quarg is prepared by inoculating milk with lactic starter culture and rennet, incubating to pH 4.6 and separating the whey by bag drainage or mechanical centrifugation. Quarg may be packaged plain or with salt, fruit preserves, spices and other condiments. It is used as a garnish for soups or salads, or served with fruit for dessert or with cereals for breakfast.

Nutrient Composition

The nutrient content of skim milk, cream and selected fermented dairy products is given in Table 14.8. All dairy products are good dietary sources of high quality protein, calcium, phosphorus, potassium and riboflavin. While many lactic organisms require B vitamins for growth, several species are capable of synthesizing vitamins. The extent of biosynthesis depends on the culture species and strain, the incubation conditions and other processing parameters.

Cottage cheese starter actively synthesized vitamin B₁₂ and folacin during the setting period. *Lactobacillus casei* used in the preparation of yakult has been shown to synthesize thiamin, riboflavin, B₆ and B₁₂.

Table 14.8 : Nutrient Composition of 100 Portions of skim Milk. Cream and Selected Fermented Dairy Products

Nutrient	Skim milk	Cream (light)	Buttermilk	Cultured cream	Plain yogurt (low fat)	Cottage cheese (low fat)
Energy (kj)	146	818	169	987	265	303
Carbohydrate (g)	4.8	3.7	4.8	4.3	7.0	2.7
Fat (g)	0.2	19.3	0.9	21.0	1.6	1.0
Protein (g)	3.4	2.7	3.3	3.2	5.2	12.4
Calcium (mg)	123	96	116	116	183	61
Iron (ug)	40	40	50	60	80	140
Magnesium (mg)	11	9	11	11	17	5
Phosphorus (mg)	101	80	89	85	144	134
Potassium (mg)	166	122	151	144	234	86
Sodium (mg)	52	40	105	53	70	406
Zinc (ug)	400	270	420	270	870	380
Ascorbic acid (mg)	1.0	0.8	1.0	0.9	0.8	trace
Thiamine (ug)	36	32	34	35	44	21
Riboflavin (ug)	140	148	154	149	214	165
Niacin (ug)	88	57	58	67	114	128
Pantothenic acid (ug)	329	276	275	360	591	215
Vitamin B ₆ (ug)	40	32	347	16	49	68
Folacin (ug)	5	2	trace	11	11	12
Vitamin B ₁₂ (mg)	0.38	0.22	0.22	0.30	0.56	0.63
Vitamin A(IU)	204	720	33	790	66	37

Others monitored the syntheses and utilization of B vitamins by three strains each of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* incubated for 24 h in skim milk. *Streptococcus thermophilus* strains 1 and 3 synthesized significant amounts of folacin. None of the strains had a significant effect on the synthesis of biotin, B₁₂ or niacin. *Lactobacillus bulgaricus* strains 1 and 2 utilized significant amounts of biotin ; strains 2 and 3 utilized folacin; strain 3 utilized niacin. None of the strains of *Lactobacillus bulgaricus* synthesized B vitamins. Cultured cream, yogurt and cottage cheese contain more than twice the level of folacin than the milk or cream from which they are made (Table 14.8).

Prehydrolysis of Milk Components

The nutritional value of a food is dependent not only on its nutritional content, but also on the availability, digestibility and assimilability of its nutrients. Thus, fermentation may increase the nutritional value of milk through the enzymatic hydrolysis of carbohydrates, fats and proteins by the lactic starter cultures.

The milk carbohydrate lactose may not be digested properly in persons who are deficient in the intestinal enzyme B-galactosidase (B-gal). Infants, children and adolescents require the calcium in milk for proper bone growth and development and a restriction in their intake of dairy products because of problems with lactose intolerance may have serious nutritional consequences. Up to 50% of the lactose in yogurt, 48% of the lactose in acidophilus milk and 26% of the lactose in buttermilk may be hydrolyzed during manufacture and storage. In addition, yogurt starter culture produces significant quantities of β -gal which may enhance the digestion of lactose when yogurt is consumed by lactose intolerant individuals. Alm reported that the consumption of acidophilus milk or yogurt caused no intestinal discomfort in a test group of lactose intolerant individuals. Recently, lactose intolerant persons consuming a non-fermented sweet acidophilus milk also demonstrated improved lactose utilization as monitored by the breath hydrogen test.

Chandan summarized previous studies on the lipolytic and proteolytic activities of various lactic cultures and noted that the streptococci and leuconostocs play an important role in the prehydrolysis of milk fat, while the lactobacilli are important in the prehydrolysis of the milk protein.

L-Glutamic Acid Fermentation

Introduction

The flavor enhancing property of konbu, a kelp-like seaweed traditionally used as a seasoning source in Japan, was identified as being due to L-glutamic acid. This discovery led to the industrial production of monosodium L-glutamate.

The annual production of monosodium L-glutamate, produced exclusively by fermentation, exceeds 370,000 tonnes. L-Glutamic acid is a seasoning in widespread use throughout the world. It is also used as a starting material for the synthesis of various kinds of speciality chemicals. *N*-Acylglutamate is commercially available as a biodegradable surfactant with low skin irritation properties which is valued as an additive in cosmetics, soaps and shampoos. Oxopyrrolidinecarboxylic acid, another derivative of L-glutamic acid, is used as a natural moisturizing factor in cosmetics, playing an important role in maintaining water in the cornified layer, acting synergistically with glycine, threonine, alanine, aspartic acid, glutamic acid and serine. Amides of acylglutamate are utilized as gelatinization agents. They render a wide variety of hydrocarbon and vegetable oils jelly-like, and have applications in oil dispersion for marine antipollution purposes.

Production of L-Glutamic Acid

Microbial Strains

A number of wild strains that have been isolated as L-glutamic acid-producing bacteria are shown in Table 1. Most of these L-glutamic acid-producing bacteria are Gram-positive, non spore-forming, non-motile and require biotin for growth. Among these strains, bacteria belonging to the genera *Corynebacterium* and *Brevibacterium* are in widespread use along with an oleic acid-

requiring auxotrophic mutant, which was derived from biotin-requiring *Brevibacterium thiogenitalis*.

Tabel 15.1 : Microbial Strains Producing L-Glutamic Acid

Genus	Species
<i>Corynebacterium</i>	<i>C. glutamicum</i> , <i>C. lilium</i> , <i>C. callunae</i> , <i>C. herculis</i>
<i>Brevibacterium</i>	<i>B. divaricatum</i> , <i>B. aminogenes</i> , <i>B. flavum</i> , <i>B. roseum</i> , <i>B. immariophilum</i> , <i>B. alanicum</i> , <i>B. ammoniagenes</i> , <i>B. thiogenitalis</i>
<i>Microbacterium</i>	<i>M. salicinovolum</i> , <i>M. ammoniaphilum</i> , <i>M. flavum car. glutamicum</i>
<i>Arthrobacter</i>	<i>A. globiformis</i> , <i>A. aminofaciens</i>

Culture Conditions

Carbon source

L-Glutamic acid-producing bacteria can utilize various carbon sources, such as glucose, fructose, sucrose, maltose, ribose or xylose, as the substrate for cell growth and L-glutamic acid biosynthesis. For industrial production, molasses and starch hydrolyzates are generally employed as the carbon source.

In order to obtain high yields of L-glutamic acid, the biotin concentration in the medium must be strictly controlled at a suboptimum level for the maximum cell growth. Therefore, biotin-rich raw materials, e.g. beet molasses and cane moases, could not be used until the discovery of the biotin mediating effects of penicillins and C₁₆–C₁₈ saturated fatty acids. Oleic acid-requiring mutants accumulate L-glutamic acid in biotin-rich media only when the oleic acid concentration is controlled at the suboptimal level for maximum growth.

Nitrogen source and pH control

The ample supply of a suitable nitrogen source is essential for L-glutamic acid fermentation, since the molecule contains 9.5% nitrogen. Ammonium salts such as ammonium chloride or ammonium sulfate are assimilable. L-Glutamic acid-producing bacteria also have a strong urease activity, so urea is also utilizable as a nitrogen source. The ammonium ion is detrimental to both cell growth and product formation, and its concentration in the medium must be maintained at a low level. The pH of the culture medium

is very apt to become acidic as ammonium ions are assimilated and L-glutamic acid is excreted. Gaseous ammonia has a great advantage over aqueous bases in maintaining the pH at 7.0-8.0, the optimum for L-glutamic acid accumulation. It serves as a pH-controlling agent and as a nitrogen source, and solves various technological problems. Automatic addition of gaseous ammonia makes precise pH control possible, and avoids the harmful effects of ammonia and the undesirable dilution of the fermentation liquid.

Oxygen supply

The biosynthesis of L-glutamic acid is an aerobic process requiring oxygen throughout the fermentation. For maximum L-glutamic acid production, control of dissolved oxygen at its optimum level is essential. Actively respiring cells will consume all the oxygen in a saturated broth (7 p.p.m.) within a few seconds, therefore oxygen must be supplied continuously to maintain the optimum dissolved oxygen concentration.

Accumulation of Other Products in Relation to Change in Culture Conditions

Lactic Acid and Succinic Acid

L-Glutamic acid-producing *Brevibacterium flavum* accumulate lactic acid and succinic acid when cultured under limited oxygen supply. As the rate of oxygen supply decreases from the condition of complete saturation to various degrees of satisfaction of oxygen requirement, the main product changes from L-glutamic acid to succinic acid, and then to lactic acid.

α -Ketoglutaric Acid

It was demonstrated that the absence of ammonium ions, but with sufficient oxygen supply resulted in the accumulation of α -ketoglutaric acid in place of L-glutamic acid. When the pH controlling agent.

L-Glutamine

L-Glutamic acid is converted into L-glutamine when the culture is performed in the presence of excess ammonium chloride at a weakly acidic pH in the presence of zinc ions. In a medium containing 40g l⁻¹ of ammonium chloride and 10 mg l⁻¹ of zinc sulfate, the cells accumulated more than 40g l⁻¹ of L-glutamine at

0.30g g⁻¹ carbon source. A high concentration of ammonium ions in weakly acidic conditions results in the production of *N*-acetyl-L-glutamine. Zinc ions are effective in decreasing the excretion of *N*-acetyl-L-glutamine in favor of L-glutamine accumulation.

Microbial Physiology of L-Glutamic Acid Fermentation

Permeability of Cell Membrane to L-Glutamic Acid in Relation to Biotin Concentration

A key compound controlling L-glutamic acid fermentation is biotin. The accumulation of L-glutamic acid is at a maximum when the biotin concentration is suboptimal for maximum growth. Excess biotin supports abundant cell growth but seriously decreases the L-glutamic acid accumulation. The critical biotin content of the cells for the accumulation of L-glutamic acid is 0.5 µg g⁻¹ of dry cells.

However, in the presence of excess biotin, the addition of penicillin, which is known to inhibit the formation of cross-links in the peptidoglycan of bacteria at the growth phase, permits cells to accumulate a large amount of L-glutamic acid. Other antibiotics, such as cephalosporin C, which also inhibits cell wall synthesis, can replace penicillin. The addition of C₁₆–C₁₈ saturated fatty acids or their esters with hydrophilic polyalcohols during the growth phase also permits cells to accumulate L-glutamic acid in a biotin-rich medium. The use of these antibiotics and C₁₆–C₁₈ saturated fatty acids allowed industrial utilization of biotin-rich raw materials such as cane and beet molasses.

The accumulation of L-glutamic acid is governed not by its biosynthesis but by its excretion. The excretion of L-glutamic acid is closely related to the cell permeability, which is associated with both chemical and physical constituents of the cell membrane. L-Glutamic acid-producing cells grown with limited biotin or grown with excess biotin and treated with either penicillin or Tween-60 excreted intracellular L-glutamic acid when washed with phosphate buffer. Cells grown with excess biotin without the treatment of penicillin or Tween-60, however, do not. This phenomenon has also been observed for L-aspartic acid. Other amino acids were washed out of the cells even when grown under biotin-rich condition. L-Glutamic acid-excreting cells, notwithstanding biotin limitation, oleic acid requirements or C₁₆–C₁₈ saturated fatty acid treatment, have a low content of

phospholipids in the cell membrane. On the other hand, the cells with a poor ability to accumulate L-glutamic acid in a biotin-rich medium have a much higher concentration of membrane phospholipids.

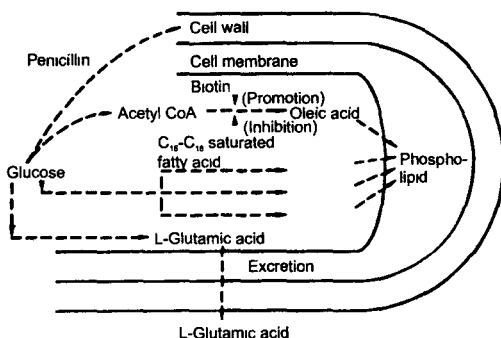


Fig. 15.1 : Cell permeability to L-glutamic acid in relation to phospholipid content in the membrane.

As shown in Figure 15.1, biotin is a cofactor of acetyl-CoA carboxylase, the enzyme in the biosynthesis of oleic acid, and C₁₆-C₁₈ saturated fatty acids inhibit the biosynthesis of oleic acid by repressing acetyl-CoA carboxylase. Limited amounts of biotin or C₁₆-C₁₈ saturated fatty acids cause incomplete biosynthesis of oleic acid resulting in a decrease in phospholipid concentration. Consequently, phospholipids such as cardiolipin and phosphatidylinositol dimannoside were thought to be involved in the regulation of the permeability of cells to L-glutamic acid.

Regulatory Mechanisms of L-Glutamic Acid Biosynthesis

Although the permeability barrier is more important, the regulatory mechanisms of L-glutamic acid biosynthesis have been studied in order to obtain mutants with increased productivity.

In the production of L-glutamic acid, two enzymes have been shown to play important roles; phosphoenolpyruvate carboxylase (PC), which catalyzes carboxylation of phosphoenolpyruvate to form oxaloacetate, and α -ketoglutarate dehydrogenase (KD), which converts α -ketoglutarate to succinyl-CoA. The efficiency of carbon dioxide fixation to give oxaloacetic acid depends on PC activity. Aspartic acid showed both inhibition and repression of the enzyme, and the inhibition was enhanced by α -ketoglutaric acid. Therefore, the endogeneous pool of aspartic acid and α -ketoglutaric acid must

be minimized if the production of L-glutamic acid is to be maximized. KD is essential for complete oxidation of glucose to carbon dioxide. This enzyme is strongly inhibited by *cis*-aconitate, succinyl-CoA, NADH, NADPH, pyruvate and oxaloacetate, while being stimulated by acetyl-CoA. The properties of KD of L-glutamic acid producing bacteria are favorable for the preferential synthesis of L-glutamic acid from α -ketoglutaric acid, preventing the further oxidation of α -ketoglutaric acid to carbon dioxide and H₂O *via* succinyl-CoA. The K_m value of KD for α -ketoglutaric acid was shown to be about one seventieth of that of L-glutamic acid dehydrogenase, which catalyzes the formation of L-glutamic acid from α -ketoglutarate. On the other hand, V_{max} of L-glutamic acid dehydrogenase was about 150 times larger than that of KD. Consequently, the endogeneous concentration of α -ketoglutaric acid, which controls the relative metabolic flow of α -ketoglutarate leading to L-glutamic acid biosynthesis or further oxidation, was shown to be high enough to overproduce L-glutamic acid preferentially.

Genetic Improvement of L-Glutamic Acid-producing Microorganisms

Initial overproduction of L-glutamic acid was performed with wild strains in which the permeability barrier was modified, but the productivity was further increased by microbial breeding. In one example, the cell's permeability barrier to L-glutamic acid was modified by mutation a temperature sensitive mutant, which showed normal growth at 30°C but little or no growth at 37°C, produced a large amount of L-glutamic acid even in a medium with excess biotin when the culture temperature was shifted from 30°C to 40°C during the cultivation. The membrane synthesis of this mutant was considered to be insufficient at 37–40°C, thus permitting L-glutamic acid excretion. No chemical control by penicillin or C₁₆–C₁₈ saturated fatty acids was needed for the overproduction of L-glutamic acid in biotin rich media.

Another attempt to improve the production yield involved increasing the carbon dioxide fixation. L-Glutamic acid is biosynthesized through the glyoxylate cycle as an oxaloacetate generating system without carbon dioxide fixation (Figure 15.2a), and through phosphoenolpyruvate to form oxaloacetate with carbon dioxide fixation (Figure 15.2b). The increase in carbon dioxide fixation might improve the production yield.

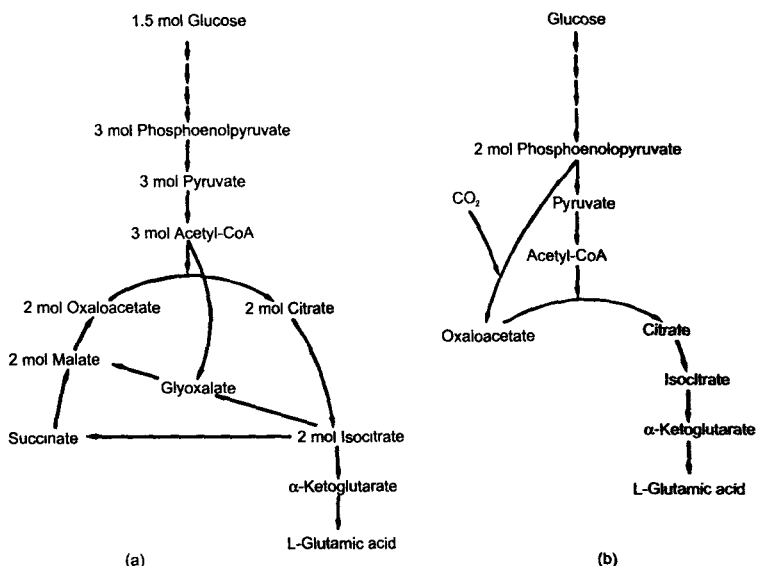


Fig. 15.2 : (a) Biosynthetic pathway of L-glutamic acid through the glyoxylate cycle as oxaloacetate generating system without carbon dioxide formation. (b) Biosynthetic pathway of L-glutamic acid through phosphoenolpyruvate as oxaloacetate generating system with carbon dioxide fixation.

Several of the monofluoroacetate resistant mutants derived from *Brevibacterium lactofermentum* showed improved productivity of L-glutamic acid in parallel with the increased PC activity. One of these mutants with decreased isocitrate lyase activity also accumulated increased amounts of L-glutamic acid. The extent of carbon dioxide fixation was increased in these improved mutants.

A pyruvate dehydrogenase leaky mutant derived from *Brevibacterium lactofermentum* utilized acetic acid and glucose simultaneously to give the higher yield, in which acetic acid was considered to be assimilated as a substrate of acetyl-CoA and glucose as a substrate of oxaloacetate.

Application of DNA recombination techniques to the improvement of L-glutamic acid-producing bacteria is a promising new route. Several kinds of plasmids of *Brevibacterium* and a plasmid of *Corynebacterium* relating to spectinomycin resistance were found to be suitable as a possible vector system. Construction

of a chimera plasmid involving a gene associated with L-glutamic acid biosynthesis was performed with *Brevibacterium lactofermentum*.

Performance of Large Scale L-Glutamic Acid Fermentations

Figure 15.3 shows a typical large scale fermentation system equipped with automatic control. Continuous rather than batchwise sterilization is more successful in eliminating undesirable foreign microbes in the large volume of the media. The advantages are (1) energy saving, (2) better quality control and (3) improved productivity. Air filters packed with glass wool are usually employed for air sterilization.

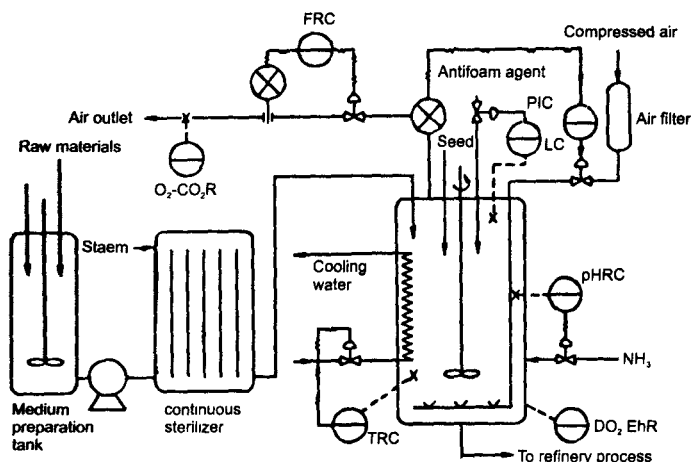


Fig. 15.3 : An example of an automatically controlled fermentation process: LC level control; PIC pressure control; pHRC pH recording and control; FRC flow rate recording and control; TRC temperature recording and control; EhR redox potential recording.

In L-glutamic acid fermentation, less power input is needed for agitation than in antibiotic fermentation as the bacterial culture fluids have a lower viscosity than mycelial culture fluids. However, it should be noted that oxygen requirement and heat evolution per unit time and unit volume of the culture are higher because of the higher rate of sugar assimilation and cell respiration.

For a successful fermentation operation, dissolved oxygen tension, temperature and pH must be optimized throughout the fermentation, the dissolved oxygen maintained above 0.01 atm by changing air flow rate, temperature controlled through a cooling

device, and culture pH maintained at a constant level by gaseous ammonia. These controls can be performed by computer-aided systems. In addition, the sequential control of many operations, for example sterilizing the system, continuous medium sterilization, introduction of the medium into the fermenter, feeding the concentrated sugar solution into the fermenter, and then washing the fermenter with water, can be easily programmed to occur synchronously.

Conclusions

Research and development of L-glutamic acid fermentation changed the commercial production method of monosodium L-glutamate from a protein hydrolysis process to a microbial production process. The protein hydrolysis method was more costly, using expensive wheat gluten or soybean protein as raw materials, and resulted in a large amount of byproducts such as starch or amino acid mixtures. L-Glutamic acid fermentation, on the other hand, does not yield any particular byproducts, and has now completely replaced the protein hydrolysis method.

Moreover, recent technological innovation, such as DNA recombination, cell fusion and bioreactor development, are now being applied for further improvement of L-glutamic acid fermentation. DNA recombination and cell fusion techniques might be useful for the genetic construction of microorganisms with higher production yields or with the capability to assimilate less expensive raw materials such as C₁-compounds and cellulosic materials. Bioreactors packed with -glutamic acid-producing microorganisms are being investigated in an attempt to improve the productivity.

16

Phenylalanine

Phenylalanine was until recently only a minor part of amino acid market. The use of L-aspartylphenylalanine as a sweetener has caused an increase in the market share of phenylalanine, as well as for aspartic acid.

Control Mechanisms of Phenylalanine Biosynthesis

Maximal production of phenylalanine by direct fermentation depends on the circumvention of the complex metabolic regulation caused by the three common products of phenylalanine synthesis: tyrosine, tryptophan and phenylalanine itself. Fundamental studies of metabolic regulation have provided effective guiding principles for the development of high yielding mutants, and the selection of phenylalanine producers of *Bacillus Breuibacterium* and *Corynebacterium* has been carried out in parallel with the investigation on the regulatory mechanism of phenylalanine biosynthesis.

The principal regulatory mechanisms for the biosynthesis of phenylalanine in *Becillus subtilis* are summarized in Figure 16.1 DAHP synthetase, the first enzyme in the phenylalanine-tyrosine tryptophan pathway, is inhibited by chorismate and prephenate, and repressed by tyrosine alone and synergistically with phenylalanine. The activity of shikimate kinase is also inhibited by both chorismate and prephenate *via* feedback control. Prephenate dehydratase, the first enzyme of the phenylalanine specific pathway, is inhibited by phenylalanine itself.

Figure 16.2 summarizes the other types of regulatory mechanism in *Brevibacterium flavum*. DAHP synthetase in the common pathway is controlled by phenylalanine and tyrosine *via* negative feedback. Anthranilate synthetase, in tryptophan synthesis, is strongly inhibited by tryptophan, whereas chorismate

mutase is free from regulation by phenylalanine and tyrosine in the synthesis of both phenylalanine and tyrosine. Prephenate aminotransferase, specific for tyrosine synthesis, is not regulated by tyrosine, while prephenate dehydratase for phenylalanine synthesis is regulated by phenylalanine.

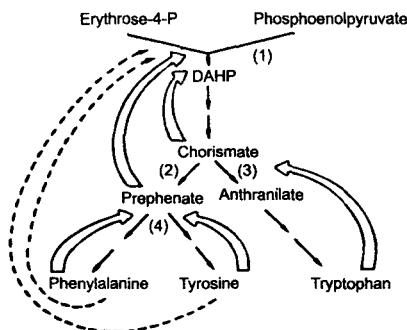


Fig. 16.1 : Regulatory mechanisms for L-phenylalanine biosynthesis in *Bacillus subtilis*. feedback inhibition; feedback repression (1) DAHP synthetase; (2) chorismate mutase; (3) anthranilate synthetase; (4) prephenate dehydratase.

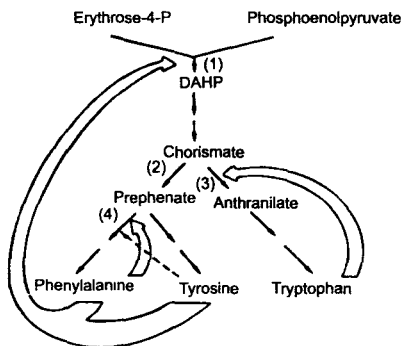


Fig. 16.2 : Regulatory mechanisms for L-phenylalanine biosynthesis in *Brevibacterium flavum*. feedback inhibition; removal of inhibition (1) DAHP synthetase; (2) chorismate mutase; (3) anthranilate synthetase; (4) prephenate dehydratase.

In *Corynebacterium glutamicum*, DAHP synthetase is inhibited synergistically by tyrosine, phenylalanine and tryptophan, the effects of single amino acids being only very slight. The activity of prephenate dehydratase is inhibited by phenylalanine and tryptophan, although stimulated by tyrosine.

Direct Fermentative Production of Phenylalanine from Sugars

Maximal production of phenylalanine in *Bacillus subtilis* depends on the desensitization of prephenate dehydratase to negative feedback by phenylalanine. A β -thienylalanine resistant mutant, whose prephenate dehydratase was desensitized and thus resistant to the feedback control, excreted about 4 g l^{-1} of L-phenylalanine. Similarly, other phenylalanine producers resistant to such phenylalanine analogs as P-fluorophenylalanine (PEP), *m*-fluorophenylalanine (MEP), *p*-aminophenylalanine (MFP), P-aminophenylalanine (PAP) and 5-fluorotryptophan (5 T) were derived; a resistant to 5 FFT produced 6 g l^{-1} of L-phenylalanine and 4 g l^{-1} of L-tryptophan.

In *Brevibacterium flavum*, the prime goal was to desensitize the DAHP synthetase and/or prephenate dehydratase to feedback inhibition by phenylalanine. MFP resistant mutants accumulated 2 g l^{-1} of L-phenylalanine. Either DAHP synthetase or prephenate dehydratase was free from feedback inhibition by phenylalanine in these mutants. The development of tyrosine auxotrophic mutants is also an effective means by which to avoid feedback inhibition of DAHP synthetase by tyrosine. The productivity of tyrosine auxotrophic mutants was about 1.6 g l^{-1} , lower than that of MFP resistant mutants. However, better producers of phenylalanine were obtained by the combination of both auxotrophic and regulatory mutants. The production of 25 g l^{-1} of L-phenylalanine was reported by a tyrosine auxotrophic, PFP and 5MT resistant mutant derived from *Brevibacterium lactofermentum*.

A tyrosine auxotrophic mutant resistant to PFP and PAP, derived from *Corynebacterium glutamicum*, also produced 9.5 g l^{-1} of L-phenylalanine in a molasses medium. Phenylalanine production in this mutant was stimulated by tryptophan but inhibited by tyrosine. The prephenate dehydratase of this mutant was resistant to inhibition by phenylalanine and tryptophan, although its DAHP synthetase still remained sensitive (Table 16.1).

Production with Phenylpyruvic Acid as a Precursor

The use of intermediates as substrates in phenylalanine synthesis avoids the inhibition by metabolites. Phenylpyruvic acid, an intermediate precursor in the biosynthesis of phenylalanine, is

Table 16.1 : Production of L-Phenylalanine by Direct Fermentation

Strain	Marker	Substrate	L-phe formed (gl ⁻¹)	Yield (%)
<i>Bacillus subtilis</i>	5FT	Glucose	6.0	7.5
<i>Brevibacterium lactofermentum</i>	5MT, PFP, Dec ⁻ , Tyr ⁻ , Met ⁻	Glucose	25.0	19.0
<i>Corynebacterium glutamicum</i>	PFP, PAP, Try	Cane molasses	9.5	9.5

converted into L-phenylalanine by *Alcaligenes*, *Pseudomonas* and *Escherichia*. For example, dried cells of *Alcaligenes faecalis* B14-1 converted 10.9 gl⁻¹ of phenylpyruvic acid into L-phenylalanine with a conversion efficiency of 78% . In this reaction, L-aspartic acid, L-leucine and L-glutamic acid were used as amino donors, and better results were obtained when these amino acids were used in combination as shown in Table 16.2. This probably means that this strain had several L-phenylalanine aminotransferases specific to each amino donor.

Table 16.2 : Performance of Certain Amino Acids as Amino Donors in L-Phenylalanine Synthesis

Amino acid	Concentration (mmol)	L-Phe formed (mg tube ⁻¹)	Yield (%)
L-Aspartate	200	9.00	54.5
L-Glutamate	200	7.67	46.5
L-Leucine	200	8.25	50.0
L-Aspartate	100	9.40	56.9
L-Glutamate	100		
L-Aspartate	70		
L-Glutamate	70	11.65	70.6
L-Leucine	70		

Enzymatic methods

Phenylalanine ammonia-lyase and hydantoinase have both been used independently for the production of L-phenylalanine. L-phenylalanine ammonia-lyase of *Sporobomyces roseus* generally catalyzes the breakdown of L-phenylalanine to phenylsuccinate and ammonia. However, this proces for L-phenylalanine production utilizes the lyase to synthesize L-phenylalanine from

phenylasaccinate and uses excess ammonium ions to reverse the reaction. The concentration of L-phenylalanine obtained using this method was 28 g l^{-1} and the production yield was 80% on a molar basis.

L-Phenylalanine has also been enzymatically synthesized from DL-5-indolylmethylhydantoin. A wild strain of *Flavobacter aminogenes*, which hydrolyzes DL-5-indolylmethylhydantoin to L-tryptophan, was used for the formation of L-phenylalanine from DL-5-indolylmethylhydantoin. The hydantoinhydrolyzing enzyme of this strain was induced by DL-5-indolylmethylhydantoin. Fifty mg ml^{-1} of wet cells converted 10 mg ml^{-1} DL-5-indolylmethylhydantoin to L-phenylalanine with a yield of 100% on a molar basis. The fact that the D-form of hydantoin was racemized to its L-form by the intact cells of *Flavobacter aminogenes* was advantageous in obtaining such high reaction yields.

Isomerization of D-Phenylalanine to L-Phenylalanine

Chemical synthesis of L-phenylalanine, a method currently still in commercial use, involves isomerization of DL-phenylalanine. The asymmetric hydrolysis of acyl-DL-amino acids by microbial aminoacylase was found to be better than any other enzymatic method, and was further improved by immobilization. The reaction can be carried out continuously in columns packed with aminoacylase immobilized on a DEAE Sephadex support (Izumi *et al.*, 1978).

Indigenous Fermented Foods

Fermented foods, whether from plant or animal origin, are an intricate part of the diet of people in all parts of the world. It is the diversity of raw materials used as substrates, methods of preparation and sensory qualities of finished products that is so astounding a one begins to learn more about the eating habits of various cultures. The preparation of many indigenous or "traditional" fermented foods and beverages remains today as a household art.

Table 17.1 lists some of the more common indigenous fermented foods consumed in various parts of the world.

Fermented Foods of the Orient

Soy Sauce

The written records of the Chinese show that they have been using soy sauce for over three thousand years. Production of soy sauce in Japan probably was a result of the introduction of Buddhism from China and the consequent change to a vegetable diet in 552 A.D. The technology of soy sauce preparation was at one time a closely guarded family art passed on from one generation to the next. While there are still unique formulae used on a domestic level, the major steps involved in the manufacture of soy sauce are no longer a secret. There is, however, much to be learned about the biochemical changes which occur during fermentation and lead to desirable as well as undesirable sensory qualities in the finished product.

Two distinct basic processes can be used to prepare soy sauce. The first involves fermentation with microorganisms and the second, i.e., chemical method, involves the use of acids to promote hydrolysis of ingredient constituents. The latter method will not be

discussed here mainly because it cannot be considered as traditional or indigenous, but also because there are some who consider the end product to be inferior and not in a class deserving of recognition as a substitute for the fermented product.

The koikuchi soy sauce represents the largest amount prepared in Japan; approximately 90% of the production is of this type. It is an all-purpose seasoning characterized by a strong aroma and dark reddish-brown color. A second type of soy sauce, amounting to less than 10% of the production, is called Usukuchi. It is lighter in color, milder in flavor, and is used mainly for cooking when preservation of the original flavor and color of the foodstuffs is desired. Tamari style soy sauce produced in china traditionally. represents about 2% of the production. This soy sauce is characterized by a strong flavor and dark brown color. Produced in lesser amounts are Saishikomi soy sauce which is characterized by a high level of reducing sugars and a yellowish tan color. All of these types contain relatively high levels of salt, in the range of 17 to 19% and all are used as seasoning agents to enhance the flavor of meats, seafoods and vegetables. Known as shoyu in Japan, soy sauce is called chiang-yu in China, kecap in Indonesia, kanjang in Korea, toyo in the Philippines and see-ieu in Thailand .

1. Preparation of soybeans

A flow sheet for manufacturing soy sauce is shown in Fig. 17.1. Two processes, viz., soaking and cooking soybeans, and roasting and crushing (cracking) wheat, are separate yet simultaneous, in the early stages of production. Whole soybeans or defatted soybean meal or flakes can be used in the production of soy sauce. If whole beans are used, oil must eventually be removed from the fermented mash: otherwise an inferior product will result. This oil is used for making a low-grade soap or as a source of linoleic acid pressed or, more commonly, solvent-extracted soybean meal is used. Cost is lower, utilization of nitrogen is higher, and fermentation time is shorter with defatted beans than with whole soybeans. This may be due to a lower surface: volume ratio in whole beans versus meal, hence a more pronounced physical restraint in whole beans with regard to the access of enzymes and organism to soybean components during fermentation. Oil from whole soybeans which rises to the surface of the mash may also eventually retard microbiological activity during aging.

Table 17.1 : Indigenous Fermented Foods^{a)}

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Ang-kak (anka, red rice)	China, Southeast, Asia, Syria	Rice	<i>Monascus purpureus</i>	Dry red powder	Colorant
Bagoong	Philippines	Fish	Unknown	Paste	Seasoning agent
Bagni	Caucasus	Millet	Unknown	Liquid	Drink
Banku	Ghana	Maize, cassava	Lactic acid bacteria, yeasts	Dough	Staple
Bonkrek	Central Java (Indonesia)	Coconut press cake	<i>Rhizopus oligosporus</i>	Solid	Roasted or fried in oil, used as a meat substitute
Bouza	Egypt	Wheat	Unknow	Liquid	Thick acidic
Braga	Romania	Millet	Unknown	Liquid	Drink
Burukutu	Savannah region of Nigeria	Sorghum and casava	Lactic acid bacteria, <i>Candida</i> spp., <i>Sacch- aromyces cerevisiae</i>	Liquid	Creamy drink with suspended solids
Busa	Tartars of Krim, Turkestan, Egyp	Rice or millet, sugar	<i>Lactobacillus</i> and <i>Saccharomyces</i>	Liquid	Drink
Chee-fan	China	Soybean whey curd	<i>Mucor</i> sp. <i>Aspergil lus glaucus</i>	Solid	Eaten fresh, cheese-like
Chicha	Peru	Maize	<i>Aspergillus</i> , <i>Penicil lium</i> spp., yeasts. bacteria	Spongy	Eaten with vegetables
Chickwangue	Congo	Cassava roots	Bacteria	Paste	Staple

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Chinese yeast	China	Soybeans	Mucoraceous molds and yeasts	Solid	Eaten fresh or canned, used as side dish with rice
Darassum	Mongolia	Millet	Unknown	Liquid	Drink
Dawadawa (daddowa, uri, Kapalugu, kinda, neteton)	West Africa, Nigeria	African locust bean	Lactic acid bacteria, yeasts	Solid. sun-dried	Eaten fresh, supplement to soups, stews
Dhokla	India	Bengal gram and wheat	Unknown	Spongy	Condiment
Dosai (doza)	India	Black gram and rice	Yeast, <i>Leuconostoc mesenteroides</i>	Spongy, pancakelike like	Breakfast food
* Compiled from BEUCHAT (1978). HESSELTINE (1979). HESSELTINE and WANG (1980). REDDY et al. (1982), and STANTON and WALLBRIDGE (1969)					
Fish sauce (Nuoc-man, patis, mampla, ngamplyye)	Southeast Asia	Fish	Bacteria	Liquid	Seasoning agent
Gari	West Africa	cassava rot	<i>Corynebacterium manihot</i> , <i>Geotrichum candidum</i>	Wet paste	Eaten fresh as staple with stews, vegetables
Hamanatto	Japan	Whole soybeans, wheat flour	<i>Aspergillus oryzae</i> , <i>Streptococcus</i> , <i>Pedococcus</i>	Beans retain individual from raisin-like, soft	Flavoring agent for meat and fish. eaten as snack

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Idli	Southern India	Rice and black gram	Lactic bacteria (<i>Leuconostoc mesenteroides</i>). <i>Torulopsis candida</i> and <i>Trichosporon pullulans</i>	Spongy, moist	Bread substitute
Injera	Ethiopia	Teff, or maize wheat, barley, sorghum	<i>Candida guilliermondii</i>	Bread-like, moist	Bread substitute
Jalebies	India, Nepal, Pakistan	Wheat flour	<i>Saccharomyces bayanus</i>	Pretzel-like	Bread substitute
Jamin-bang	Brazil	Maize	Yeasts and bacteria	Bread or cake-like	Bread substitute
Kaanga-kopuwai	New Zealand	Maize	Bacteria and yeasts	Soft, slimy	Eaten as vegetable
Kanji		India	Rice and carrots	<i>Hansenula anomala</i>	Liquid Sour, added to vegetables
Katsuobushi	Japan	Whole fish	<i>Aspergillus glaucus</i>	Solid, dry	Seasoning agent
Kecap	Indonesia and vicinity	Soybeans, wheat	<i>Aspergillus oryzae</i> , <i>Lactobacillus</i> , <i>Hansenula</i> , <i>Saccharomyces</i>	Liquid seasoning agent	Condiment,
Kenima	Nepal, Sikkim, Darjeeling district of India	Soybeans	Unknown	Solid	Snack
Kenkey	Ghana	Maize	Unknown	Mush	Steamed, eaten with vegetables
Ketjap	Indonesia	Black soybeans	<i>Aspergillus oryzae</i>	Sirup	Seasoning agent

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Khaman	India	Bengal gram	Unkown	Solid, cake-like	Breakfast food
Kimchi (Kim-chee)	Korea	Vegetables, sometimes seafoods, nuts	Lactic acid bacteria	Solid and liquid	Condiment
Kishk	Egypt, Syria, Arab world	Wheat, milk	Lactic acid bacteria, <i>Bacillus</i> spp.	Solid	Dried balls dispersed rapidly in water
Lafun Lao-chao	West Africa, Nigeria China, Indonesia	Cassava root Rice	Bacteria <i>Rhizopus oryzae</i> , <i>R. chinensis</i> , <i>Chlamydomucor oryzae</i> , <i>Saccharomycopsis</i> sp.	Paste Soft. Juicy. glutinous	Staple food Eaten as such as dessert or com- bined with eggs. seafood
Mahewu (Magou)	South Africa	Maize	Lactic acid bacteria (<i>Lactobacillus delbruckii</i>)	Liquid	Drink, sour and non-alcoholic
Meitauza	China, Taiwan	Soybean cake	<i>Actinomucor elegans</i>	Solid	Fried in oil or cooked with vegetables
Meju	Korea	Soybeans	<i>Aspergillus oryzae</i> <i>Rhizopus</i> spp.	Paste	Seasoning agent
Merissa	Sudan	Sorhum	<i>Saccharomyces</i> sp.	Liquid	Drink
Minchin	China	Wheat gluten	<i>Paecilomyces</i> , <i>Aspergillus</i> , <i>Cladosporium</i> , <i>Fusarium</i> ,	Solid	Condiment

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Miso(chang, jang, doenjang, tauco, tao chieo)	Japan, China	Rice and soybeans or rice and other cereals such as barley	<i>Syncephalastum</i> , <i>Penicillium</i> , <i>Trichothecium</i> spp. <i>Aspergillus oryzae</i> <i>Torulopsis etchellsii</i> , <i>Lactobacillus bacteria</i> , <i>Saccharomyces rouzii</i>	Paste	Soup base, seasoning
Munkoyo	Africa	Millet, maize or kaffir corn plus roots of munkoyo	Unknown	Liquid	Drink
Nan(khab-z)	India, pakistan, Afghanistan, Iran	Unbleached wheat Flour	Unknown	Solid	Snack
Natto	Norhern Japan	Soybeans	<i>Bacillus natto</i>	Solid	Cake, as a meat substitute
Ogi	Nigeria, West Africa	Maize	<i>Lactic bacteria</i> , <i>Cephalosporium</i> , <i>Fusarium</i> , <i>Aspergillus</i> , <i>penicillium</i> spp., <i>Saccharomycetes cerevisiae</i> , <i>Candida mycoderma</i> (<i>C.ralida</i> or <i>C.rini</i>)	Paste	Staple, eaten for breakfast. weaning babies
Oncom (ontjom, lontjom)	Indonesia	Peanut press cake	<i>Neurospora intermedia</i> , less often <i>Rhizopus oligosporus</i>	Solid	Roasted or fried in oil, used as meat substitute

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Papadam	India	Black gram	Saccharomyces spp.	Solid. crisp	Condiment
Peujeum	Java	Banana, plantation	Unknown	Solid	Eaten fresh or fried
Pito	Nigeria	Guineacorn or maize or both	Unknown	Liquid	Drink
Poi	Hawaii	Taro corms	<i>Lactobacillus bacteria</i> , <i>Candida vini</i> (<i>Mycoderma vini</i>) <i>Geotrichum candidum</i>	Semi solid	Side dish with fish. meat
Pozol	Southeastern Mexico	Maize	Molds, Yeastes, bacteria	Dough, spongy	Diluted with water, drunk as basic food
Prahoc	Cambodia	Fish	Unknown	Paste	Seasoning agent
Puto	Philippines	Rice	Lactic acid bacteria, <i>Saccaromyces cerevisiae</i>	Solid	Sanck
Rabdi	India	Maize and buttermilk	Unkonwn	Semi-solid	Mush, eaten with vegetables
Sierra rice	Ecuadlor	Unhusked rice	<i>Aspergillus flavus</i> , <i>A.candidus</i> , <i>Bacillus subtilis</i>	Solid	Brownish-yellow. seasoning

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
Sorghum beer (Ibantu beer, kaffir beer, Ieting, joala,	South Africa	Sorghum, maize	Lactic acid bacteria yeasts	Liquid	Drink, acidic and weekly alcoholic
Soybean milk	China, Japan	Soybeans	Lactic acid bacteria	Liquid	Drink
Soy sauc (Chaing-yu, shoyu, toyo, kanjang, kecap, seeieu)	Japan, China, Philippines, other parts of Orient	Soybeans and wheat	<i>Aspergillus oryzae</i> or <i>A. soyae</i> , <i>Lactobacillus</i> bacteria, <i>Saccharomyces</i> <i>rouxii</i> (<i>Zygosaccharo-</i> <i>myces</i> spp.)	Liquid	Seasoning for meat fish, cereals, vegetables
Suf (tahuri, taokaoan, tao-hu-yi)	China, Taiwan	Soybean whey curd	<i>Actinomucor elegans</i> , <i>Mucor hiemalis</i> , <i>M. silvaticus</i> , <i>M. subtilissimus</i>	Solid	Soybean cheese. condiment
Tao-si	Philippines	Soybeans plus wheat flour	<i>Aspergillus oryzae</i>	Semi solid	Seasoning agent
Taotjo	East Indies	Soybeans plus wheat meal or glutinous rice	<i>Aspergillus oryzae</i>	Semi solid	condiment
Tape	Indonesia and vicinity	Cassava or rice	<i>Saccharomyces</i> <i>cerevisiae</i> , <i>Hansenula</i> <i>anomala</i> , <i>Rhizopus</i> <i>oryzae</i> , <i>Chlamydomucor</i>	Soft solid	Eaten fresh as staple

contd.....

Table 17.1 – contd.....

Product	Geography	Substrate	Microorganisms (s)	Nature of Product	Product Use
			<i>oryzae</i> , <i>Mucor sp.</i> <i>Endomycopsis fibuliger</i> (<i>Saccharo mycopsis</i> <i>sp.</i>)		
Tarhan	Turkey	Parboiled wheat meal and yoghurt(2:1)	Lactic acid bacteria	Solid powder	Dried seasoning for soups
Tauco	West Java (Indonesia)	Soybeans, cereals	<i>Rhizopus oligosporus</i> , <i>Aspergillus oryzae</i>	Liquid	Drink
Tempeh (temple kedeke)	Indonesia and vicinity, Surinam	Soybeans	<i>Rhizopus spp.</i> , principally <i>R. oligosporus</i>	Solid	Fried in oil. roasted or used as meat substitute in soup
Thumba (Bojah)	West Bengal	Millet	<i>Endomycopsis fibuliger</i>	Liquid	Drink, mildly alcoholic
Torani	India	Rice	<i>Hansenula anomala</i> , <i>Candida guillier mondii</i> , <i>C. tropicalis</i> , <i>Geotrichum</i> <i>candidum</i>	Liquid	Seasoning for vegetables
Waries	India	Black gram flour	<i>Candida spp.</i> , <i>Saccharomyces spp.</i>	Spongy	Spicy condiment eaten with vegetables, legumes, rice

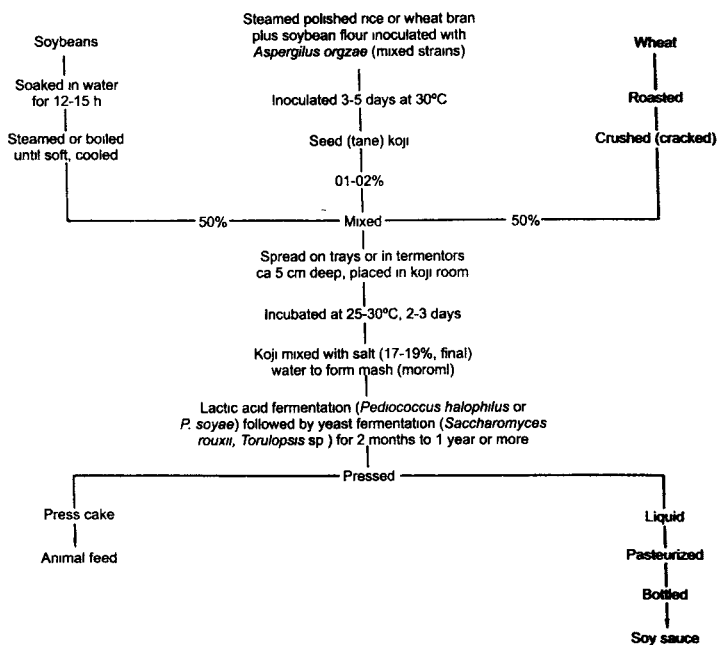


Fig. 17.1 : Flow diagram for manufacture of soy sauce.

Whole beans or meal are soaked for 12 to 15h at ambient temperature or, preferably, at about 30°C until a 2.1 times increase in weight results. Soaking is done either by running water over the beans or by changing still water every 2 or 3 h. If water is not changed, spore-forming *Bacillus* may proliferate to levels eventually deleterious to end product quality. Also, depending upon the depth of soybeans in the water, those in the bottom layer of tanks may heat if water is not changed or circulated around them during soaking. The swollen beans or meal are then drained, covered with water again and steamed to achieve further softening and pasteurization. If pressure is used, the beans can be sterilized during the cooking process. On a smaller scale, beans are boiled in an open pan until swollen beans are boiled in a smaller scale, beans are boiled in an open pan until soft enough to easily press flat between the thumb and finger. The conditions for cooking soybeans not only play an important role in influencing digestibility by enzymes during fermentation but also may affect the turbidity of the final product

when it is diluted or heated for home use. This turbidity is caused by insolubilization of undenatured protein when dispersed in concentrated salt solution and deflates the commercial value of soy sauce. With increased moisture or pressure during steaming, the soy protein tends to denature more readily: on the other hand excessively denatured soy protein has reduced accessibility for enzyme reaction. Consequently, the yield of soluble nitrogen and other soluble compounds will be reduced. Thus, the procedure used for cooking soy-bean is critical to fermentation patterns and end product quality.

Rapid cooling on an industrial scale is done by spreading the beans in about a 30 cm layer on tray-like platforms and forcing air through them. It is important to reduce the temperature to less than 40°C within a few hours: otherwise, proliferation of microorganisms may ensue and thus spoil the beans before controlled fermentation can be initiated.

2. Preparation of wheat

Concurrent with the preparation of soybeans is the roasting and crushing (cracking) of wheat. Wheat flour or wheat bran may be used in place of whole wheat kernels. The roasting of wheat contributes to aroma and flavor of soy sauce. Characteristic breakdown and conversion products produced by cooking wheat include the guaiacyl series compounds, such as vanillin, vanillic acid, ferulic acid, and 4-ethylguaiacol. The free phenolic compound content increases with heating due to degradation of lignin and glycosides. Roasting causes the desired visual properties in the finished product.

3. Koji process

The word koji, meaning "bloom of mold" refers to the enzyme preparation produced on cereals or sometimes pulses and used as a seed or starter for larger batches of plant seed substrated when making a number of traditional fermented foods. In the case of soy sauce, seed(tane) koji is produced by culturing a number of mixed strains of *Aspergillus oryzae* or *A. sojae* on either steamed, polished rice (usual practice in Japan) or a mixture of wheat bran and soybean flour (China) It is added to a soybean/ wheat mixture at a rate of 0.1 to 0.2% to produce what is then simply called koji.

Koji infected with *Rhizopus* or *Mucor* species indicates that atmospheric moisture may have increased to a level which caused water droplets to be formed on the surface at some point during incubation. Excessively contaminated Koji should be discarded because of the undesirable flavor and aroma it will impart to soy sause.

4. Mash (Moromi) Stage

When the koji is mature, it ready for brining. The koji is mixed with an equal amount or more (up to 120% by volume) of saline water to form the mash or moromi. The sodium chloride content of the mash should range from 17 to 19%. Concentrations less than 16% salt may enable growth of undesirable putrefactive bacteria during subsequent fermentation and aging. On the other hand, concentrations in excess of 23% may retard the growth of desirable osmophilic yeasts and halophilic bacteria. the mycelium of the koji mold is killed during the very early stages of mash preparation. If fermentation is allowed to proceed naturally without controlling temperature a period of 12 to 14 months is necessary for the fermentation and aging process. If the mash is kept in large wooden or concrete containers such as those used by commercial manufactures. The temperature is usually maintained at 35 to 40°C thus reducing the fermenttion and aging period to 2 to 4 months. Regardless of the storage temperature, it is important to stir the mash intermittently. This is done with a wooden stick on a small scale or with compressed air in modern commercial facilities.

During the early stages of fermentation, koji enzymes hydrolyze proteins to yield peptides and free amino acids. Starch is converted to simple sugars which in turn are fermented by microorganisms to yield lactic, glutamic and other acids as well as alcohols and carbon dioxide. As a consequence, the pH of the mash drops from near neutrality to 4.5 to 4.8. Stirring must be correlated to a considerable extent with the rate of carbon dioxide production. Elevated levels of carbon dioxide will enhance the growth of certain anaerobic microorganisms which may impart undesirable flavor and aroma to the finished product. Excessive aeration, on the other hand, will hinder proper fermentation.

The microbiology of mash is not clearly understood; however, it is known that various groups of bacteria and yeasts predominate in sequence during the fermentation and aging process. The

halophilic bacterium, *Pediococcus halophilus*, grows readily in the first stage of fermentation, converting simple sugars to lactic acid and causing a drop in pH. Later, *Saccharomyces rouxii*. *Torulopsis* and other yeasts predominate.

Table 17.2 : Some Flavor Components in Soy Sauce

Acetaldehyde	Benzoic acid
Acetone	2-Acetyl furn
Propanal	Benzaldehyde
2- Methylpropanal	Furfuryl acetate
3-Methylbutanal	2-Methyl propanoic acid
Ethyl acetate	Bornyl acetate
2-Hexanone	4-Pentanolide
2,3-Hexanedione	Butanoic acid
Ethanol	Phenyl acetaldehyde
2- Propanol	Furfuryl alcohol
2-Methyl-1-propanol	Ehyl benzoate
3-Methylbutyl acetate	3-Methylbutanoic acid
1-Butanol	Diethyl succinate
3-Methyl-1-1-Butanol	Borneol
2-Methyl-3tetrahydrofuranone	3-Methylthio-1-propanol (methional)
2-Methylpyrazine	Ethyl phenylacetate
3-Hydroxy-2- butanone (acetion)	2-Phenylethyl acetate
2,6-Dimethylpyrazine	2-Methoxyphenol (guaiacol)
2,3-Dimethylpyrazine	Benzyl alcohol
Ethyl-2 hydroxy-Propanoate (ethyl lactate)	2-Phenylethanol
2-ethyl-6-methylpyrazine	3-Hydroxy-2-methyl-4-pyrone (maltol)
Acetic acid	2-Acetylpyrrole
3-Ethyl-2,5-dimethylpyraine	2-Methoxy-4-ethylphenol (4-ethylguaracol)
Furfural	4-Ethylphenol
4-Hydroxy-2-ethyl-5-methyl-3(2H)-furanone	2,6-Dimethoxyphenol
4-Hydroxy-5-ethyl-2-methyl-3(2H)-furanone	Ethyl myristate
4-Hydroxy-5 methyl-3(2H)-furanone	

Like most traditional fermented foods, soy sauce owes its pleasant aroma or flavor largely to the activities of microorganism. A partial list of flavor components identified in soy sauce is shown in (Table 17.2). *Pediococcus halophilus* and, perhaps, *Lactobacillus* species produce lactic and other organic acids which, in themselves, contribute to aroma and flavor. However, it is the yeasts that probably make the greatest contribution to characteristic sensory qualities of soy sauce.

5. Pasteurization

The raw soy sauce is pasteurized at 70 to 80°C, thus killing the vegetative cells of microorganisms. Enzymes are also denatured, and proteins are coagulated. Alum or kaolin may be added to enhance clarification. The sauce is then filtered, bottled and marketed, preservatives are often added to prevent growth of yeasts during storage. Butyl-P-Hydroxybenzoate at a concentration of 50 µg/mL or, alternatively, sodium benzoate at 200 µg/mL are most widely used. Preparation of soy sauce in the home does not usually include pasteurization and certainly does not involve the use of chemical preservatives other than salt present as an integral part of the product.

Advances in fermentation technology have enabled manufacturers to produce sauce with consistent quality. The use of pure culture inocula at all stages of production has reduced the risk of carrying unwanted contaminants from one batch to the next.

B. Miso

Fermented soybean pastes, known by various names, are prepared using a variety of procedures in the Orient. The prototype of these pastes is believed to have been introduced in China in 600 A.D. or before. Today, fermented soybean pastes are known as *chiang* in China, *jang* or *doenjang* in Korea, *miso* in Japan, *tauco* in Indonesia, *tao chieo* in Thailand and *tao si* in the Philippines. Most *chiang* in China is prepared at home, just as people in Western countries make their own jams, jellies or pickles. In Japan, however, *miso* is now manufactured commercially in modernized factories on a large scale.

Fermented soybean pastes are consumed in various ways. *Chiang* is used as a base for sauce served with meat, poultry, sea

food and vegetable dishes. In Japan, miso is mainly used as a base for soups. The average annual per capita consumption of miso in Japan is 7.2 kg, 80 to 85% of this being consumed in the preparation of miso soup and the balance used as seasoning for other foods

Methods for manufacturing miso differ somewhat, but the basic process is the same as that shown for rice miso in (Fig. 17.2). Rice miso is made from rice, soybeans and salt; barley miso is made from barley, soybeans and salt; and soybean miso is made from soybeans and salt (Table 17.4). The three major types of miso are further classified on the basis of degree of sweetness and saltiness. The procedure for making miso consists of four major steps, two of which are carried out concurrently. These consist of preparation of the koji and the soybeans (simultaneous processes), brining or fermentation and finally aging. Each step for the preparation of rice miso will be considered separately here in order to illustrate the general procedure for making miso.

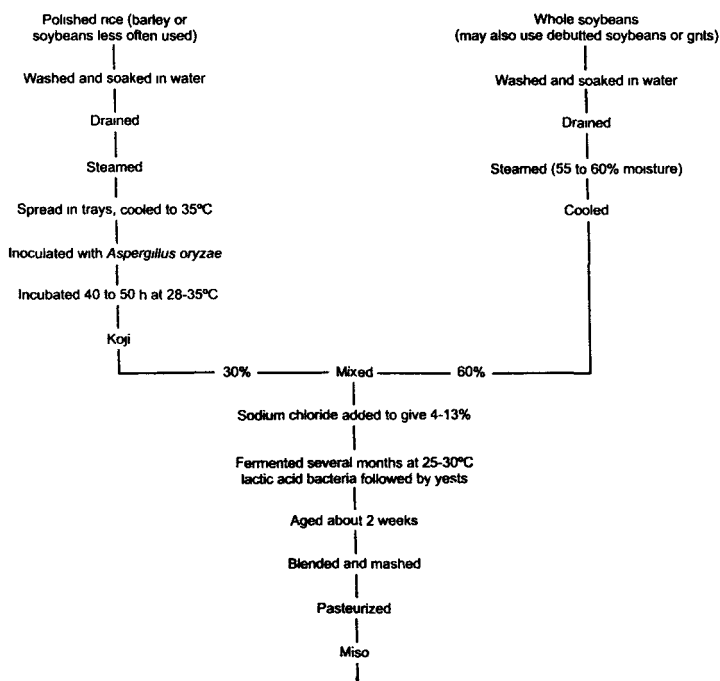


Fig. 17.2 : Flow diagram for manufacture of miso.

Table 17.3 : Types and Composition of Major Types of Miso Made in Japan^{a)}

<i>Raw Material</i>	<i>Taste</i>	<i>Color</i>	<i>Fermentation Aging Time</i>	<i>Protein</i>	<i>Fat</i>	<i>Composition (%)</i>		<i>Moisture</i>
						<i>Carbohydrate</i>	<i>Ash</i>	
Rice	Sweet	Yellowish white	5-20 days	11.1	4.0	35.9	7.0	42.0
		Reddish brown	5-20 days	12.7	5.1	31.7	7.5	43.0
	Semisweet	Bright light yellow	5-20 days	13.0	5.4	29.1	8.5	44.0
		Redish brown	3-6 months	11.2	4.4	27.9	14.5	42.0
	Salty	Bright light yellow	2-6 months	13.5	5.9	19.6	14.0	47.0
		Reddish brown	3-12 months	13.5	5.9	19.1	14.5	47.0
Barley	Semisweet	Yellowish/reddish brown	1-3 months	11.1	4.1	29.8	13.0	42.0
	Salty	Reddish brown	3-12 months	12.8	5.2	21.1	15.1	46.0
Soybeans	Salty	Dark reddish brown	5-20 months	19.4	9.4	13.2	13.0	45.0

1. Preparation of koji

Polished rice is used to prepare koji, the source of enzymes to hydrolyze soybean components later in the fermentation process; brown rice is not suitable for growth of the koji mold (*Aspergillus oryzae*) because the surface texture is hard and contains waxes which inhibit penetration of the kernel by mycelium. After washing, the rice is soaked in water overnight at about 15°C to bring the moisture content to about 35%. Excess water is removed and the rice is steamed at atmospheric pressure for 40 min to 1 h. This may be done in a batch process or by using a continuous cooker. The cooked rice is spread out on large trays or platforms to cool to about 35°C. Seed koji, prepared as described for use in soy sauce manufacturing, is added at a rate of 1 g per kg of rice. This application rate is based on a viable spore count of 10^9 per g of seed koji. The use of koji rooms in which trays of inoculated rice are stacked has been replaced largely by modern rotary fermentors. Rice is put into a large drum of the fermentor in which the temperature, air circulation and atmospheric relative humidity is controlled within critical limits. The drum is rotated to prevent the rice from agglomerating during fermentation. The temperature is maintained between 30 and 35°C to promote growth of *A. oryzae* and maximum production of saccharolytic and proteolytic enzymes.

A source of undesirable microorganisms may also be found on the surface of cooling trays and in the air surrounding the cooked rice and fermenting koji. Ventilation should be adequate to supply sufficient oxygen and to dissipate carbon dioxide, and humidity should be such that neither drying nor sticking of the rice occurs. Fermentation is complete after 40 to 50 h.

The next morning, rice is spread in wooden trays and stacked. During an approximate 50h fermentation period, rice must be stirred thoroughly to maintain uniform aeration, temperature and moisture.

Regardless of the system used to prepare koji, when fermentation is adequate, the rice is well covered with white mycelium of *A. oryzae*. Harvesting is done before sporulation with consequent pigmentation development. The product at this point is characterized by a sweet aroma and flavor; musty odors indicate an inferior quality koji. The addition of salt to koji as it is removed from fermentors or trays assures against further growth of the mold.

2. Preparation of soybeans

Concurrent with the preparation of koji is the preparation of whole soybeans for fermentation. Ideally, soybeans with thin, glossy, light yellow or white seed coats are used. The soybeans should also be large and uniform in size and have an ability to absorb water and cook very rapidly. After extraneous materials are removed by mechanical equipment or by hand, soybeans are washed and soaked in water for 18 to 22 h. Water should be changed during the soaking period, especially in the summer months when temperatures are elevated, to control the proliferation of bacteria. At the end of the soaking period, beans have increased in volume by about 240% and in weight by 220 to 260% depending upon the ratio of protein and carbohydrate.

After being drained, the soybean are cooked in water or steamed at 115°C for about 20 min in a closed cooker until they are sufficiently soft to be easily pressed flat between the thumb and finger. Flavor and color development can be achieved by varying the heating time and temperature.

3. Fermentation and aging

Cooked, cooled beans are then mixed with salted koji and inoculum. In the past, a portion of miso from a previous batch was used as the inoculum, but modern technology involves the use of starter containing pure cultures of osmophilic yeasts and bacteria. Strains of *Saccharomyces rouxii* (formerly *Zygosaccharomyces* spp.), *Torulopsis*, *Pediococcus halophilus*, and *Streptococcus faecalis* are the most important yeasts and bacteria in miso fermentation. The mixture, known as green miso, is packed into vats or tanks to undergo anaerobic fermentation and aging at 25 to 30°C for various periods of time, depending upon desired characteristics of the end product. White miso takes about 1 week, salty miso 1 to 3 months, and soybean miso over 1 year. The green miso is transferred from one vat to another at least twice to improve fermentation. The aged miso is then blended, mashed, pasteurized and packaged.

The type of miso produced is largely dependent upon the proportions of koji, salt and cooked soybeans used, but not on the type of inoculum. Thus, white miso contains 50% or less soybeans, whereas yellow or brown miso contains 50% or more soybeans with the balance being rice and salt. White miso contains 4 to 8% salt

which permit rapid fermentation, and yellow or brown miso contains 11 to 13% salt. Moisture content ranges from 44 to 52%, protein from 8 to 19%, carbohydrate from 6 to 30%, and fat from 2 to 10%, depending on the levels of soybeans, rice and barley used as ingredients. The proximate composition of various types of miso is shown in Table 17.4.

A large part of the soybean protein is digested by proteases produced by *Aspergillus oryzae* in the koji. Amino acids and their salts, particularly sodium glutamate, contribute to flavor. The addition of commercial enzyme preparations to enhance fermentation has met with some success. EBINE observed that when an enzyme preparation from a mold was added to cooked soybeans, mixed with brine and allowed to react, an improvement in miso flavor resulted. The time of fermentation was reduced by 33% and yield was increased by 8%.

Miso contains 0.6 to 1.5% acids, mainly lactic, succinic and acetic. Some esters are formed with ethyl and higher alcohols which, together with fatty acid esters derived from fatty acids of soybean lipid, are important in giving miso its characteristic aroma. Changes in lipid components, fatty acid composition and total tocopherol content during miso making were studied. Total tocopherol content was decreased in the cooking process but was unaffected during aging. Substantial hydrolysis of triglycerides was detected during the early stages of fermentation. EBINI noted that miso has strong antioxidative activity. This activity was attributed in part to the existence of isoflavones, tocopherols, lecithin, compounds of amino-carbonyl reactions and living microbial cells which tend to have a reducing activity.

There are only low levels of vitamins in miso. Cooking and steaming reduce thiamin and riboflavin levels. However, some commercial manufactures may fortify miso by adding vitamin A, thiamin and riboflavin.

4. Modified indigenous procedure

Recently there has been interest in developing miso with reduced salt content. This is due largely to a concern about the relationship between salt intake and its adverse effects on individuals suffering from high blood pressure. Ethanol was found to inactivate some enzymes such as peptidase and inhibit the

growth of microorganisms. In another study the hydrolytic activities of traditional koji and a commercial enzyme preparation from *Aspergillus oryzae* in low-salt miso were compared. Total and free amino acid levels and color intensity increased in accordance with a reduction of salt concentration. However, no marked difference was observed in the patterns of free and bound amino acids between conventional soybean miso and low salt miso, sensory evaluation revealed that a bitter taste was detected in low-salt miso, but there was no significant difference in the taste of miso soups prepared from low-salt and regular miso when the former preparation was supplemented with salt. This indicates that the bitter taste of soybean miso is suppressed by salt.

The use of *Rhizopus oligosporus* instead of *Aspergillus oryzae* as a koji mold and peanuts in place of soybeans was also studied. The total microbial population of miso during the fermentation period was altered by salt level in miso containing *R. oligosporus* koji but not in miso containing *A. oryzae*. Significantly higher populations of *Saccharomyces rouxii* were noted in low-salt (6%) miso. Changes in color occurred earlier during fermentation in low-salt miso than in high salt 12% miso, but salt level had no effect on viscosity and soluble solids. Salt level had no apparent effect on fatty acid profiles or free fatty acids, but low-salt miso had higher soluble nitrogen and free amino acid content compared to high-salt miso. It was concluded that good quality low-salt miso can be prepared using *R. oligosporus* as a koji mold and peanuts as the oilseed ingredient.

C. Fermented Whole Soybeans (natto products)

Natto is a Japanese name given to fermented whole soybeans. Similar products are known as tou-shin or tu-si by the Chinese, tao-tjo by the East Indians and tao-si by the Filipinos. The color, aroma, and flavor of these products vary, depending upon the microorganisms used to ferment the soybeans; however, natto products are generally dark in color, having a pungent but pleasant aroma and often a harsh flavor due to the relatively high level of free fatty acids. Fermented whole soybeans are eaten with boiled rice or they can be used as a seasoning agent with cooked meats, seafoods and vegetables. They are thus served as a condiment much in the same fashion as one would serve soy sauce or mustard.

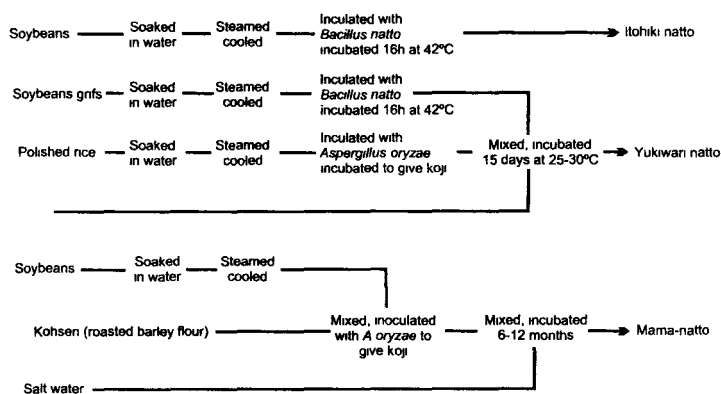


Fig. 17.3 : Flow diagram for manufacture of various types of natto.

There are three major types of fermented whole soybeans prepared in Japan. (Fig. 17.3) Itohiki-natto is produced in large quantities in Eastern Japan and is referred to simply as "natto". Washed soybeans are soaked overnight or until they are approximately doubled in weight due to uptake of water. The beans are then steamed for about 15 min, a process which serves to inactivate a large portion of the natural microbial load, and inoculated with *Bacillus natto*, a variant strain of *B. subtilis*. The beans are packaged in approximately 150-g quantities and allowed to ferment at 40 to 45 °C for 18 to 20 h. During fermentation, *B. natto*, produces polymers of glutamic acid which cause the surface of the final product to have a viscous appearance and texture. High quality natto characteristically is covered with a large amount of very viscous polymers.

A second type of fermented whole soybeans produced in Japan is known as Yukiwari-natto. This product is made by mixing Itohiki-natto with salt and rice koji, and then aging at 25 to 30°C for about 2 weeks. As with soy sauce and miso, the mold of choice for preparing the rice koji is *Aspergillus oryzae*.

Hama-natto is the third major type of fermented whole soybean product in Japan, where it is limited to an area in the vicinity of Hamanatsu. Soybeans are soaked in water for about 4 h and then steamed without pressure for 1 h. The cooled soybeans are inoculated with a koji prepared from roasted wheat and barley and fermented for about 20 h or until covered with the green mycelium of *A. oryzae*. After drying in the sun or by forced warm air to lower

the moisture to about 12%, the beans are submerged in a salt brine along with strips of ginger and allowed to age under pressure for 6 to 12 months. Extensive breakdown of proteins, carbohydrates and lipids occurs during the aging process which contributes to desirable sensory qualities of Hama-natto. In addition to enzymes originating from *A. oryzae* in the koji and which are known to hydrolyze soybean components, enzymes produced by bacteria such as *Micrococcus*, *Streptococcus*, and *Pediococcus* which are reported to be widely distributed on the surface and on the inner part of Hama-natto may also contribute to hydrolysis of soybean components.

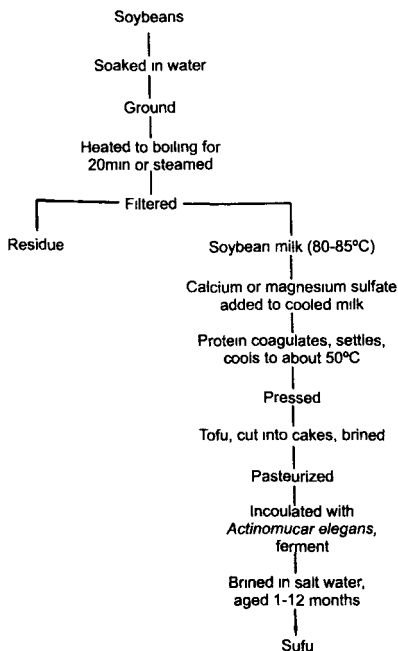


Fig. 17.4 : Flow diagram for preparing sufu.

D. Sufu

Sufu is a mold-fermented soybean curd product consumed in the Orient, particularly among the Chinese, who also refer to it as fu-ju or tou-fu-ju. Because of the difficulties of phonetic translation from Chinese to English, many synonyms for sufu have appeared in the literature. These include Tou-fu, fu-su, toe-fu, tou-fu-fu, and fu-yu. The product is known as chao in Vietnam, tahuri in the Philippines, taokaoan in Indonesia, and tao-hu-yi in Thailand.

The preparation of sufu consists of three major phases, viz, making a soybean milk curd, fermenting the curd with an appropriate mold(s) and finally brining the fermented curd (Fig.17.4). The curd is prepared in a fashion essentially the same as that used to prepare tofu. Soybeans are washed, soaked overnight and ground with water; a water to dry soybean ratio of about 10:1 is commonly used. After boiling or steaming for 20 to 30 min, the ground mass is strained through a fine metal screen to separate the

soybean milk from the insoluble residue. Alternatively, beans may be soaked, ground and strained without heating; milk is then heated to boiling to inactivate trypsin inhibitors and to reduce some of the undesirable beany flavor. Curdling of the milk is achieved by adding calcium sulfate or magnesium sulfate, and occasionally acid. The curd is then transferred to a cloth-lined wooden box and pressed with a weight to remove the whey. The finished soybean curd (tofu) contains approximately 80 to 85% water, 10% protein and 4% lipid. The Asian people attach a similar degree of importance to soybean milk and curd as people from dairy countries attach to cow's milk. Tofu is important from a nutritional standpoint because it constitutes a needed source of calcium for the diet. It is bland in flavor and as such can be flavored with soy sauce or miso, or cooked with meat, seafood, vegetables or soup.

Tofu is prepared for fermentation by cutting into 3-cm cubes and soaking in a brine containing about 6% sodium chloride and 2.5% citric acid for 1 h. This treatment retards or prevents the growth of bacterial contaminants but has little effect on growth of desired molds during subsequent stages of sufu preparation. The brined cubes are heated at 100°C for about 15 min, cooled and placed on a perforated tray in such a way that they are not touching. The cubes are surface-inoculated with a selected mold and incubated at 12 to 25°C for 2 to 7 days, depending upon the type and rate of growth of the mold and the desired flavor characteristic of the final product. The freshly molded cubes are covered with white to yellowish-white mycelium, known as pehtze; they contain 74% water, 12% protein and 4.3% lipid.

The last stage of making sufu involves brining and aging. Depending upon the desired flavor and color, pehtzes may be submerged in salted, fermented rice or soybean mash, fermented soybean paste or a solution containing 5 to 12% sodium chloride in rice containing 10% ethanol. Red rice and soybean mash impart a red color to sufu. Brines containing high levels of ethanol result in a product having a marked alcoholic bouquet. This product is known as tsui-fang or tsue-fan, which means drunk sufu. Flavor may be modified by adding hot pepper or rose essence to the aging brine. In addition to imparting a salty taste, sodium chloride also causes the release of mycelial enzymes which penetrate the molded cubes and hydrolyze soybean components. The aging period ranges from 1 to 12 months at which time the sufu is consumed as

a condiment or used in cooking vegetables or meat. On a commercial scale, sufu is bottled in the same brine and heat-sterilized before marketing.

E. Meitauza

The solid waste collected from ground, steeped, strained soybeans in the preparation of tofu and sufu is sometimes fermented to give a product known as meitauza. Soybean cakes approximately 10 to 14 cm in diameter and 2 to 3 cm thick are allowed to ferment for 10 to 15 days with moderate aeration. The best meitauza is prepared during the cooler months of the year because high temperatures may result in growth of undesirable bacteria. During the fermentation period, cakes become covered with white mycelium of *Mucor meitauza*, which is a synonym of *Actinomucor elegans*, the principal mold involved in sufu fermentation. At the end of fermentation, cakes are partially sun-dried and sold on the market. Meitauza is cooked in vegetable oil or with vegetables as a flavoring agent.

F. Lao-chao

Lao-chao, also known as chiu-niang or tien-chiu-niang by the Chinese, is a fermented rice product. Glutinous rice is first steamed and cooked, then mixed with a small amount of commercial starter known by the Chinese as chiu-yueh or peh-yueh.

The mass is incubated at ambient temperature for 2 to 3 days during which yeasts hydrolyze the starch, rendering the product soft, juicy, sweet, fruity and slightly alcoholic (1 to 2%). Filamentous fungi may also play a role in lao-chao preparation. Members of the mucoraceous fungi, including *Rhizopus oryzae*, *R. chinensis* and *Chlamydomucor oryzae* can be consistently isolated from lao-chao.

Endomycopsis, one of the few yeasts capable of producing amylases and utilizing starch, is also an integral part of the necessary microflora.

Lao-chao is consumed as such or it may be cooked with eggs and served as a dessert. Believed to help them gain strength, it has a unique place in the diet of new mothers.

G. Ang-kak

Red rice (ang-kak, ankak, anka, ang-quac, beni-koji, aga-koji) has been used in the fermentation industry for preparation of red

rice wine (Shao-Hsing wine) and foods such as sufu, fish sauce, fish paste, and red soybean curd, a cheese-like product used as a spice, for many centuries in China. pigments produced by *Monascus purpureus* and *M. anka* on a rice substrate have been used as household and industrial food colorants in many Oriental countries. Ang-kak may be marketed for these uses in the form of dried red rice or as its powder.

We described procedured for preparing ang-kak on a laboratory scale. Rice is first washed, soaked in water for about 1 day and drained thoroughly. The moist rice is then transferred to a glass beaker or suitable container to allow plenty of air space above it and autoclaved for 30 min at 121°C. Upon cooling, the rice is inoculated with a sterile water suspension of ascospores removed from a 25-day-old culture of *M. purpureus* grown on sabouraud agar. At the time of inoculation, the rice should appear rather dry. A wet or mushy substrate is undesirable. The inoculated rice is thoroughly mixed and then incubated at 25 to 32°C for about 3 days. By this time the rice will have taken on a red color and should be stirred and shaken to redistribute the moisture and kernels with respect to depth from the surface of the fermenting mass. It may be necessary to add some sterile water to replenish moisture lost during incubation. Within about 3 weeks, the rice should take on a deep purplish red color and kernels should not stick together After drying at 40°C, the kernels are easlily crumbled by slight force and may be reduced to a powder before using in foods or beaverage.

Corn can be used as a substrate for producing red pigment. However, non-glutinous varieties of rice are most suitable for preparing ang-kak, since kernels of glutinous varieties tend to stick together and thus reduce the surface to volume ratio of solid material which is so critical to pigment production. Large quantities of hydrolytic enzymes such as a amylase, β -amylase, glucoamylase, protease, and lipase are produced by *Monascus spp.* which break down the rice constituents during growth and penetration of mycelium into the kernels.

The main pigments produced by *Monascus spp.* are monascorubrin, rubropunctatin, monascin, anka-flavin, rubropunctamine, and monascorubramine (Fig. 17.5) Monascorubrin (red) and monascin (yellow) pigments produced by *M. purpureus* have probably been studied most extensively. Several researchers have invstigated the conditions necessary for good

pigment production. The optimum cultural conditions for the production of pigment by a *Monascus* sp. isolated from the solid koji of Kaoliang liquor were found to be pH 6.0 for a 3-day incubation at 32°C. Among the carbon sources tested, starch, maltose and galactose were found to be suitable for pigment production; a starch content of 3.5% (5% rice powder) and a sodium nitrate or potassium nitrate content of 0.5% gave maximum yield of pigment in laboratory media. In a later study it was shown that mutation by treatment with N-methyl-N-nitro-N nitrosoguanidine and successive isolation could be combined to improve the yield of pigment. It was reported that zinc may act as a growth inhibitor of *M. purpureus* and concomitantly as a stimulant for glucose uptake and the synthesis of secondary metabolites such as pigments.

In addition to its value as a colorant ang-kak may also possess therapeutic properties. Ailments and diseases purportedly cured by ang-kak included indigestion, bruise of muscle, dysentery and anthrax.

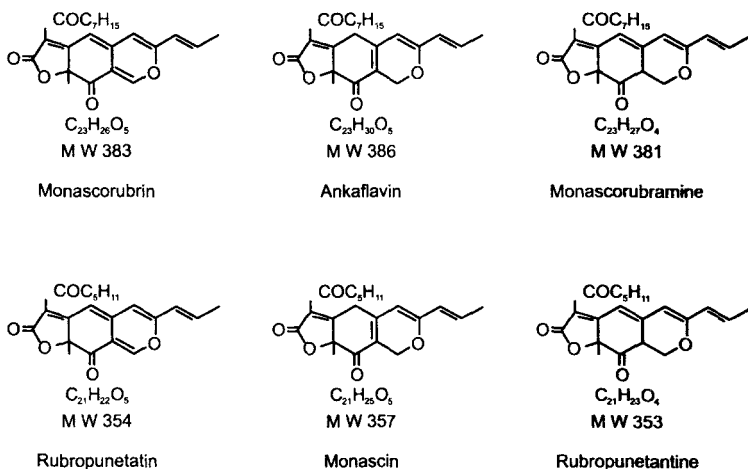


Fig. 17.5 : Molecular structures of pigments produced by *Monascus purpureus* in ang-kak.

Several species of *Bacillus*, *Streptococcus aureus*, *Pseudomonas eisenbergii* and *P. fluorescens* were inhibited by a pale yellow compound given the name monascidin by its discoverers. The minimum effective dose was about 1.5 µg per 6-mm paper assay disc. Production of the antibiotic was usually accompanied by increased pigment production.

H. Puto

A fermented rice cake commonly called puto in the Philippines is usually prepared from one-year-old rice which is ground with sufficient water to allow fermentation before steaming (Fig. 17.6). The product is similar to toyfu prepared from rice in Thailand and to idli prepared from rice and black gram mungo (*Phaseolus mungo*) in India. The quality of puto is dependednt upon the microflora present in the milled rice as well as the variety of rice used as a starting material. In an attempt to determine the influence of rice composition on puto-making qualities, we evaluated the organoleptic characteristics, volume expansion and fermentation activities of twelve rice varieties. There was a high degree of correlation ($r = 0.704$) between amylose content (within limits of 20 to 27%) and general acceptability. For texture and flavor, there was no correlation with amylose content; however good correlation ($r = 0.846$) was observed between high amylose content and satisfactory volume expansion. Varieties having very fine texturā characteristics tended to be sticky whereas coarse-textured varieties tended to be dry.

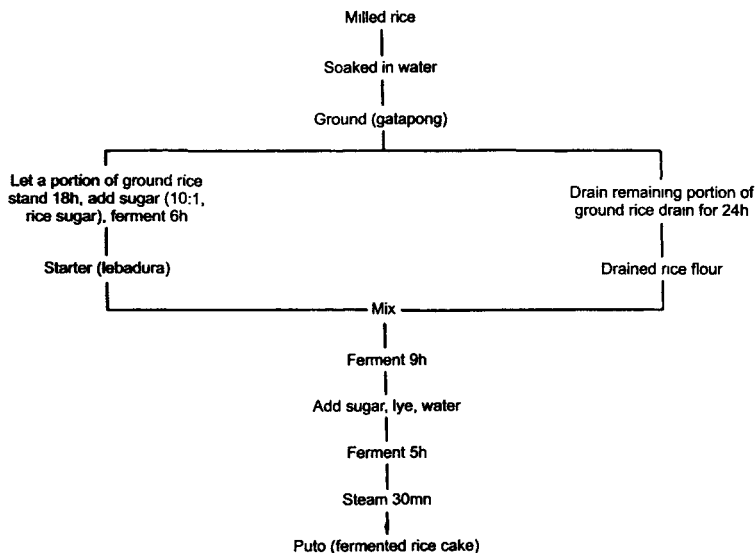


Fig. 17.6 : Flow digram for preparing puto.

A method for shortening the approxi-mately 42 h required for preparing puto by the traditional process was later reported. This

was accomplished with the use of a starter containing *Leuconostoc mesenteroides*, *Streptococcus faecalis* and *saccharomyces cerevisiae*. Sensory evaluation showed that there was no significant difference in general acceptability, texture, flavor, and traditional and shortened (21 h) methods.

I. Ragi

The Indonesian word "ragi" is roughly equivalent to the English word yeast, but is somewhat wider in scope, since it may include filamentous fungi as well,

and connotes the starter or inoculum used to initiate various kinds of fermentations. Thus, for example, Indonesian bread (roti) is made with the use of a baker's yeast preparation (ragiroti), fermented glutinous rice or cassava products called tape-ke-ton and tape-ke-tella, respectively, are prepared using ragi-tape, and tempeh is prepared using ragi-tempeh. Each type of ragi has its own mycological profile (Table 17.4). Some fungi are common to more than one type of ragi, while others are not.

Table 17.4 : Mycological profile of Various Types of Ragi produced in Indonesia

Type of Ragi	Fungus
Roti	Saccharomyces cerevisiae Candida solani
Tempeh	Rhizopus oryzae R. arrhizus R. oligosporus R. stolonifer Mucor rouxii M. javanicus
Tape	Candida parapsilosis C. melinii C. lactosa H. anomal H. malang Chlamydonucor oryzae Aspergillus oryzae
Ketjap	Rhizopus oligosporus R. arrhizus Aspergillus oryzae

Ragi is a source of enzymes necessary for the breakdown of carbohydrates and proteins in grains, legumes and roots used as main fermentation substrates. KATO et al. (1976) isolated a strain of this yeast from ragi-tape and found that it produced α -D-1,4 gluconogluco-hydrolase (EC 3.2.1.3). It releases the β -form of glucose by hydrolysis and has specific activities toward maltodextrins with four degrees of polymerization, amylose, amylo-ectin and glycogen but little or no activity toward α -methyl- or p-nitrophenyl- α -glucoside. Glucamylase of *E. fibuliger* may be one of the principal enzymes involved in saccharification of rice and cassava starch in the fermentation of tape.

J. Tape

Indonesian tape ketan is a fermented, partially liquefied, sweet-sour, mildly alcoholic rice paste. In the traditional process, fermentation is initiated by the addition of powdered ragi made from rice flour containing the desired fungi. In a practical sense, yeasts and molds naturally present in the environment and on equipment used to manufacture tape serve as inocula for preparig ragi.

Tape ketan (tepej) is prepared by fermenting glutinous rice (*Oryza sativa glutinosa*) whereas tape ketella (Indonesian), tape telo (Javanese) or peujeum (Sundanese) are prepared by fermenting cassava roots (*Manihot utilisima*). Rice or peeled, chopped cassava is steamed or cooked until soft, spread in thin layers in bamboo trays, inoculated with powdered ragi, covered with a banana or other suitable leaf and allowed to ferment for 1 to 2 days. At this time, the product will take on a white appearance, soft texture and pleasant, sweet alcoholic aroma and flavor. Both products may be consumed as such or in the case of tape ketella, fried in oil before consumption.

Amylomyces rouxii (formerly *Chlamydomucor oryzae*) in combination with eight yeasts that had been isolated from ragi-tape, with particular emphasis on *Endomycopsis burtonii* (Syn.: *E. chodati*), were evaluated. *Amylomyces rouxii* used about 30% of the total rice solids, resulting in a crude protein content of 12% in 96 h, whereas a combination of *A. rouxii* and *E. burtonii* reduced total solids by 50% in 192 h, causing crude protein to increase to 16.5%. The mold alone reduced the starch content of rice from 78 to 10% in 48 h and to less than 2% in 144 h; the mold plus yeast reduced the starch content to about 18% in 48 h. The thiamin content of the rice increased nearly threefold as a result of fermentation by *A. rouxii* in combination with *E. burtonii*. The mold and at least one species of yeast were required to develop the rich aroma and flavor of typical tapé-ketan.

A mixture of *chlamydomucor oryzae* and *Endomycopsis fibuliger* originally isolated from Indonesian ragi had good fermentation characteristics. Prepared starters, produced by growing the fungi on rice and then dehydrating them, were as active as cultures grown on a synthetic agar medium. Little change in activity of the *C. oryzae* starter was observed after 5 months of

storage at 20°C. Thus, technologies have been developed for a modernized industrial process.

Brem and arak are prepared in a fashion similar to that for tape, but the fermentation period is longer, resulting in a greater liquefaction of rice. The liquid portion is sundried to form a sweet solid product known as brem or can be rehydrated and consumed as a mild alcoholic beverage known as arak.

K. Tempeh

A popular fermented soybean product in Indonesia, New Guinea, and Surinam is tempeh (tempe). Kedelee or kedele, meaning soybean, is used to differentiate tempeh made using soybeans from tempeh bongkrek, a product prepared from coconut press cake (copra). Tempeh kedele is preferred to the less costly tempeh bongkrek. For purposes of simplification, the word tempeh as used in the following text will be synonymous with tempeh kedele.

1. Preparation of soybeans and fermentation

Tempeh is made by fermenting dehulled soybeans with various *Rhizopus* species (Fig. 17.7). The traditional process is simple and rapid, although considerable variations exist. Soybeans are soaked in water at ambient temperature overnight or until hulls (testa) can be easily removed by hand. Others prefer to parboil the beans before soaking in water. After the hulls are removed, cotyledons may be pressed slightly to remove more water and then mixed with small pieces of tempeh from a previous batch or *ragi tempeh*, a commercial starter. The inoculated beans are then spread onto bamboo frames, wrapped in banana leaves and allowed to ferment at ambient temperature for 1 to 2 days. At this point, the soybeans are covered with white mycelium and bound together as a cake

A somewhat more elaborate method for preparing tempeh involves first washing and boiling soybeans (an operation taking 2 h), transferring to cold water and soaking for about 1 day. The hulls are removed and the cotyledons are boiled again and then steamed. Meanwhile the tempeh mold is prepared by wrapping a

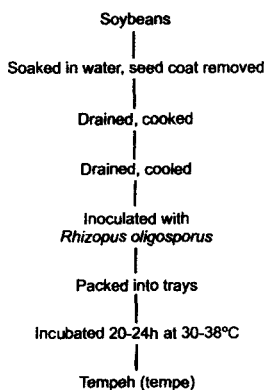


Fig. 17.7 : Flow diagram for preparing tempeh.

small portion of tempeh in a teak leaf and allowing it to incubate and dry for 2 days. The inoculum is then cut into small pieces and sprinkled over the soybeans which are in turn wrapped in banana leaves and left to ferment for about 1 day.

In a devised methods to make tempeh rapidly in large amounts by pure-culture fermentations in shallow wooden and metal trays with perforated bottoms and covers. Excellent tempeh was also made in perforated plastic bags and tubes. These methods for preparing tempeh can be carried out within a 24 h period at 31°C.

The method involves the use of lactic acid as an acidifier in the soak water. After dehulling, cleaned soybeans are returned to the acidified soak water and boiled for 90 min, drained, cooled to about 37°C and inoculated with *Rhizopus*. Acidification of the soak water to about pH 5.0 was suggested as a procedure for inhibiting the growth of some microorganisms which can cause spoilage. Ko (1970), on the other hand, purposely inoculated tempeh with various populations of *Escherichia coli*, *Bacillus mycoides*, *Pseudomonas pyocyanea*, *P. cocovenenans*, and *Proteus* sp. and concluded that the presence of these bacteria did not interfere with fermentation.

The shelf life of tempeh can be prolonged by various methods. In Indonesia, freshly prepared tempeh is sliced and then sun-dried. It was ported on a pilot plant process they developed for dehydrating tempeh by a hot air dryer at 93°C for 90 to 120 min and some described the acceptability and stability of tempeh preserved in sealed cans for 10 weeks. Acceptability was not significantly changed when the can was stored at -29°C immediately after sealing or when the tempeh was packed in water, steam-vacuum sealed, heat processed at 115°C for 20 min and stored at room temperature. The acceptability of tempeh tended to decrease during storage if tempeh had been air-dried at 60°C for 10 h prior to sealing in a can and storing at room temperature.

2. Biochemical Changes

Although other genera of molds are occasionally found in tempeh, none of them in pure culture can produce tempeh except species of *Rhizopus*. These include *R. oligosporus*, *R. stolonifer*, *R. arrhizus*, *R. oryzae*, *R. formosensis*, and *R. achlamydosporus*.

Although *Rhizopus oligosporus* cannot utilize flatulence-causing oligosaccharides because it does not produce α -

galactosidase compared with unfermented soybean grits, tempeh made from grits has been reported to have a delayed effect on gas formation in humans. Delay was attributed to possible inhibition of intestinal microflora by an antibiotic substance produced by *Rhizopus*.

The hemicellulose content (as glucose) is reduced from 2.8% in raw soybeans to 2.0% as the beans are cleaned and cooked, and to 1.1% after fermentation. The fiber content, however, may actually increase upon fermentation due to the production of mycelium by the mold.

Protease production by *R. oligosporus* is substantial, and perhaps plays the most important role in developing good quality tempeh.

The lipid content of soybeans is reduced (on a dry weight basis) during the soaking process. The temperature of the soak water may influence the rate of release of lipid from soybeans. There may be a slight decrease in lipids as a result of fermentation.

Lipid in tempeh has been found to be more resistant to autoxidation than that in raw soybeans, indicating the presence of an antioxidant as a result of fermentation. The peroxide value of lyophilized tempeh stored at 37°C for 5 months increased from 6 to 12 compared with 6 to 246 in non-fermented beans. The phytic acid content of soybeans is reduced by about one-third as a result of fermentation with *Rhizopus oligosporus*. While an equivalent amount of phosphate was released in the tempeh. The pH optimum of phytase produced by the mold was 5.6.

3. Nutritional characteristic

It was observed the beneficial effects of tempeh on patients suffering with dysentery in the prison camps of World War II. They suggested that tempeh was much easier to digest than non-fermented soybeans; however, animal feeding studies have not confirmed this conclusion. The PER of tempeh is not changed substantially from that of raw soybeans but is reduced upon frying in oil; steaming for 2 h has no effect on the other hand, reported that diets containing soybeans fermented with *Rhizopus oligosporus* had a greater apparent net protein utilization and apparent biological value than did non-fermented soybeans. Tempeh-like products containing peanuts and wheat place of part of the soybean have PER values as good as or better than 100% soybean tempeh.

Niacin, riboflavin, pantothenic acid, and vitamin B₆ contents of tempeh are reported by some researchers to be higher than levels in raw soybeans. Riboflavin is three to five times higher and niacin increases by a factor of 3.4. We stated that tempeh made with a pure culture of *R. oligosporus* contains very little vitamin B₁₂ compared with tempeh made by traditional methods. Apparently a bacterium present in commercial samples of tempeh produces vitamin B₁₂ during fermentation. Indonesian tempeh may contain 30 µg/g of B₁₂.

It was found that *R. oligosporus* produces an antibacterial agent which is especially active against some Gram positive bacteria, including both microaerophilic and anaerobic types, e.g., *Streptococcus cremoris*, *Bacillus subtilis*, *Staphylococcus aureus*, *Clostridium perfringens* and *C. sporogenes*.

L. Oncom

Oncom (onchom, ontjom, lontjom) is a fermented peanut press cake product prepared and consumed largely in Indonesia. It is consumed as a snack or for breakfast. The flavor of fermented peanut press cake is fruit-like and somewhat alcoholic but takes on a mincemeat or almond character if the product is deep-fried. Oncom may also be roasted, covered with boiling water and seasoned with salt or sugar before it is eaten. In another fashion, the roasted product is cut into pieces and covered with a ginger-flavored sauce before eating.

1. Preparation of peanuts and fermentation

The general scheme for preparing oncom is illustrated in (Fig. 17.8). After oil has been extracted from peanuts, the press cake, called boongkil, is broken up and soaked in water for about 24 h. Technical grade press cake is low in residual oil content while village products contain considerable amounts. Oil that rises to the surface of the water during the soaking period is removed and the press

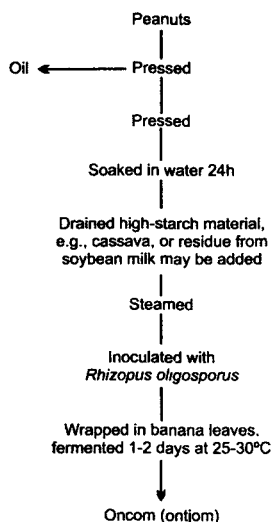


Fig. 17.8 : Flow diagram for preparing oncom.

cake is then steamed for 1 to 2 h and pressed into a layer about 3 cm deep in a bamboo frame. The mass is inoculated with either *Neurospora intermedia* (formerly *N. sitophila*) or *Rhizopus oligosporus*, the same mold used to prepare tempeh. *Neurospora* is a common mold in woody material under tropical rain forest conditions whenever sterilization or pasteurization has occurred, such as in the burning of forest land and presumably would be available to the Indonesian villager for use in inoculating a new batch of oncom. More often, as is commonly done in preparing traditional fermented foods, a small portion of a previous batch of oncom can be used as an inoculum.

The inoculated press cake is covered with banana leaves and allowed to ferment for 1 to 2 days, at which time the internal portion of the mass has been invaded by mycelia. Constant aeration is important in the production of oncom, as are temperature moisture content and degree of press cake granulation. Although covered with banana leaves, the oncom is exposed to the air during fermentation. This practice aids in sporulation of the mold inoculant, thus resulting in an orange to apricot-colored product if *N.intermedia* is used or a grey to black product if *R.oligosporus* is used. Only the surface of the fermented press cake is covered with colored conidios-pores or sporangiospores. A temperature range of 25 to 30°C is suitable for producing the best oncom.

A high-carbohydrate material such as tapioca (cassava) or potato peels may be added to the press cake prior to inoculation to enhance fermentation. It was found cultures of *Neurospora* isolated from oncom grew well on a substrate with a pH lower than 6.0. They concluded that extraction of peanut press cake with hot water, addition of 1% tapioca followed by pasteurization and adjustment of pH to 4.5 resulted in the best substrate for growth, sporulation, color, and flavor development. The addition of tapioca to peanuts has been shown to promote the growth of *Rhizopus* sp. and the addition of citric acid (1.25% by weight), tapioca (1% and sodium chloride (0.63%) to defatted peanut flour has been demonstrated to enhance the growth of *Neurospora intermedia*, *Rhizopus oligosporus*, *R.delemar* and *Aspergillus* spp. Salt may exert a beneficial osmotic or ionic effect on fungal mycelium, whereby extracellular enzymes are readily freed to act upon substrate constituents. *Mucor dispersus*, a mold used in sufu fermentation, has been reported to have increased proteinase activity when sodium chloride is added to a soybean substrate.

2. Biochemical changes

Both *Neurospora intermedia* and *Rhizopus oligosporus* produce considerable amounts of hydrolytic enzymes which act upon peanut constituents during fermentation.

Qualitative changes in proteins resulting from hydrolytic activities of *Neurospora intermedia* and *Rhizopus oligosporus* and seven other molds used to ferment foods. Large molecular weight globulins were hydrolyzed to smaller components and the percentages of specific amino acids and proportions of specific amino acids within the free amino acid varied greatly among the ferments as well as between fermented and unfermented peanut substrates. The percentages of total amino acids as free in peanuts fermented for 98 h at 28°C were reported as 8.67 and 2.99 for *N.intermedia* and *R.oligosporus*, respectively.

Lipid is hydrolyzed by the oncom molds as a consequence of fermentation of peanuts. At 28°C, as much as 9.4 and 31.5% of the lipid can be in the form of free fatty acids as a result of fermentation with *N. intermedia* and *R.oligosporus*. respectively. The free fatty acid fraction contains a significantly higher level of saturated fatty acids, particularly palmitic and stearic acids, and lower levels of linoleic acid than does the total lipid of oncom. Differences in free fatty acid distribution are those which would be expected from the action of 1.3-lipases, since saturated acids are located primarily in the 1.3 position and linoleic acid is in the 2-position of peanut triglycerides. It is well known that flavor development in fermented dairy products is significantly influenced by lipolytic activities of various microorganisms employed. Undoubtedly, lipid hydrolysis by *N. intermedia* and *R.oligosporus* also enhances the flavor and aroma of oncom.

The phytic acid content of unfermented peanut press cake was reduced from a level of 1.36% on a dry weight basis to 0.70% by *Neurospora* and 0.05% by *R.oligosporus* after 72 h of fermentation. Penta-, tetra-, tri-di- and monophosphates of inositol, as well as inorganic phosphate and inositol, as well as inorganic phosphate and inositol, were found in the fermented cake, Phytic acid is nutritionally important because of its ability to form insoluble complexes with zinc and thereby reduce its bioavailability. Thus the degradation of phytic acid by oncom molds may contribute to an overall improvement in nutritional quality of raw peanut press cake.

M. Fish Products

Fermented fish sauce and paste are popular condiments prepared and consumed in the Orient. Whole small fish, with or without entrails, or shrimp are heavily salted (up to 30% sodium chloride), packed into containers and allowed to undergo fermentation for periods ranging from a few days to over a year. Roasted cereals, glutinous or red rice flour and bran may be added in varying amounts to prepare fish pastes. The process for making fish sauce and paste is essentially anaerobic and involves bacterial and autolytic breakdown of proteins and lipids in fish tissue to result in highly flavored products which complement an otherwise bland rice diet.

Because of their high salt content, consumption of large quantities of fish sauce and paste is limited. They do, however, represent an important source of calcium for a population whose diet may be somewhat low in this mineral.

Fish sauce and paste are known as ngampya-ye and ngapi, respectively, in Burma, nuoc-mam and mams in Cambodia and vietnam, patis and bagoong in the philippines and mampla and kapi in Thailand .While traditional methods for preparing sauce or paste are similar in different countries, variations exist to result in desired appearance, aroma, texture, and flavor characteristics in the final products. For exemplary purposes, a limited number of products will be discussed here.

Nuoc-mam. Nuoc-mam, a clear brown liquid, is produced on a large scale commercially as well as in cottage industries. Total production was estimated at 40 million liters per year in the early 1970s, and daily per capita consumption ranged from 15 to 60 mL. It may be produced from small marine or freshwater fishes. On a small scale, fish are kneaded and pressed by hand, salted, tightly packed into earthenware pots, sealed and allowed to ferment. The supernatant fluid that accumulates is known as nuocmam. On a commercial scale, fish are brined in large, wooden cylindrical vats. Six parts of uncleaned fish are mixed with four to five parts salt and piled above the top of the vat. After 3 days, the liquid (nuoc-bio), which is turbid and bloody in appearance, is collected. At this point the fish are tightly packed and a portion of the nuoc-boi (now clearer in appearance due to chemical changes and a settling of solid materials) is added back to cover the mass by a depth of about 10

cm. Aging then proceeds for a few months if small fish are being fermented, or as long as 18 months for large fish.

First-quality nuoc-mam is drawn off from the matured ferment using a tap; a product having lower quality can be obtained by extract in the residual mass with fresh brine solution. The total and amino acid nitrogen content of first-quality nuoc-mam is about double that of ordinary quality product. First-quality nuoc-mam contains not less than 16 g of total nitrogen per liter; the extent of fermentation is based on formol titratable nitrogen, but not less than 50% total nitrogen; and the amount of ammonia nitrogen, an index of nutritive value, cannot exceed 50% of the formol nitrogen. One part of fish gives from two to six parts nuoc-mam, depending upon the quality of the final product.

Little is known about the contribution of microorganisms to nuoc-mam fermentation. The high level of salt greatly inhibits proteolytic activity; however, bacteria and perhaps yeasts present on and within the fish as they are packed into vats undoubtedly play some role in desired fermentation patterns and flavor development. The addition of proteolytic enzymes from *Aspergillus oryzae* to the fermenting fish has been shown to reduce the fermentation time increase the yield of nuoc-mam. Fresh pineapple and papaya have also been used to increase the rate of proteolysis.

Bagoong. Bagoong is a fish paste prepared in the Philippines from sea fish, anchovies, ambassids or shrimp. Salt is mixed with three parts fish, placed in clay vats and left undisturbed for 3 months. The resulting paste-like product is eaten raw or cooked.

Prahoc. Two stages are involved in preparing prahoc, a fermented fish paste of Cambodia. The first stage takes place at the fishing place. Fish are beheaded, eviscerated, packed in wicker baskets and tamped by foot to loosen and remove the scales. The fish are then washed in water, drained, covered with banana leaves and weighted with stones or some other heavy object for about 24 h. Salt is added to the fish at a ratio of 1:10 and the fish are then spread out to sun-dry for an additional day. The salted, semi-dried fish are again tightly packed into wicker baskets.

For the second stage of prahoc preparation, the fish are moved to homes of villagers where they are pounded into a paste, packed into earthen jars, and placed in the sun for about a month. Fluid which accumulates on the surface of the fermenting paste is

removed daily and used as nuoc mam. The prahoc is then ready for consumption, principally in the preparation of soups. Three parts of fish will produce about one part of prahoc.

Phaak. Phaak (or mamchao) is a fermented paste product produced in parts of Indochina (Cambodia) containing eviscerated, salted fish with heads and glutinous rice pretreated with yeasts. Roasted rice, with or without sugar, and ginger may also be added to obtain a special flavor in the final product. In other parts of Southeast Asia, other flavoring agents such as peppers as well as colorants may be added to fish paste to satisfy local tastes.

Katsuobushi. A fermented fish product of Japan which is neither a sauce nor a paste but rather a hard, dried product is known as katsuobushi. The product is made from skipjack tuna or bonito which is dried and allowed to mold over a period of several weeks. Ripening of katsuobushi can be hastened by artificially inoculating the fish with *Aspergillus glaucus*.

N. Kimchi

Kimchi (kimchee) is a class designation for salted and fermented products prepared in Korea. Winter kimchi made in the fall contains cabbage (Chinese and Korean), large-rooted radishes, leeks, onions, garlic, red pepper, ginger, and salt. The mixture is allowed to undergo fermentation with lactic acid bacteria for several weeks or months before being used summer kimchi is made from young radishes and radish leaves, cucumbers, cabbage, lettuce and some of the above minor ingredients, and is fermented only 2 or 3 days before serving. Small, salted shrimp, ground raw fish or nuts may also be a part of the kimchi formula.

Kimchi serves as a supply of preserved vegetables, particularly during the winter months when fresh green vegetables are less abundant. The consumption per person per day has been estimated to be 200 to 300 g in Korea. Kimchi is regarded as an important source of ascorbic acid and may represent a vehicle to supply vitamin B₁₂ if the bacteria responsible for fermentation can be selected and controlled identified some of the anaerobic bacteria in kimchi as *Lactobacillus plantarum*, *L. brevis*, *Leuconostoc mesenteroides* and *Pediococcus cerevisiae*. The presence of *propionibacterium freudenreichii* has been shown to increase the vitamin B₁₂ content of kimchi twofold.

Fermented Foods of India

A. Idli

Idli is a steamed fermented dough made from various proportions of rice and black gram flours. It is typically eaten for breakfast and is especially popular in South India, although consumed throughout the country. Proportions of rice to black gram cotyledons used to prepare idli range from 1:4 to 4:1 depending upon taste preference and availability of ingredients. Idli containing higher amounts of rice is characterized by a more predominant starchy flavor. Other ingredients such as cashew nuts, ghee, chili peppers, ginger, fried cumin seeds or curry leaves may be added to the dough in small quantities to impart additional flavor.

A flow diagram for preparing traditional idli is shown in Fig. 17.9 dehulled black gram and rice are washed and soaked in water separately for 5 to 10 h at ambient temperature. The amount of soaking water can vary from 1.5 to 2.2 times the dry weight of the black gram or rice we found that 1.5 times water over dry ingredients was optimum for fermentation and for idli preparation. After soaking, the black gram is ground with water to give a coarse paste whereas the rice is ground to give a smooth gelatinous paste. Salt (about 0.8%) is added to the combined mixture of pastes and fermentation is allowed to proceed for 15 to 24 h. Upon steaming, the soft, spongy final product resembling a sour bread or pancake is consumed while still hot. The spongy open texture of idli is attributed to the protein (globulin) and polysaccharide (arabino-galactan) in black gram.

Acidification and leavening are the most important processes which occur during fermentation.

Idli water volume increases 1.6 to 3.1 times and the pH falls from an initial 6.0 to 4.3 during fermentation. Thus, the sour taste of idli is a necessary and desirable characteristic.

The lactic acid bacteria are obviously responsible for pH reduction in idli. They may also contribute significantly to improvement of the nutritional value of unfermented black gram and rice.

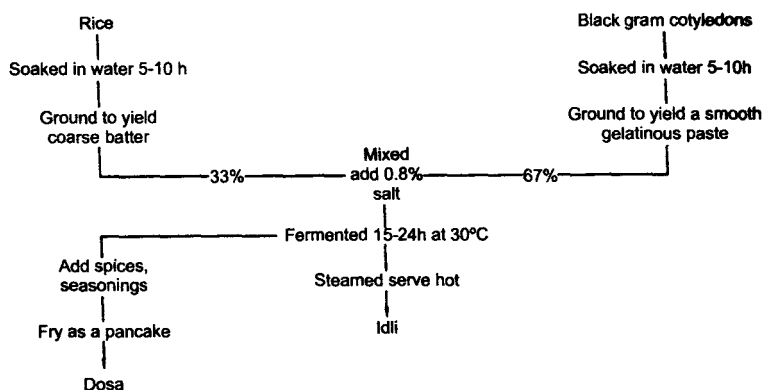


Fig. 17.9 : Flow diagram for preparing Idli and dosa. Adapted from REDDY et al.(1982).

We investigated the effects of *Leuconostoc mesenteroides*, *Lactobacillus fermenti*, *L.delbrueckii* and *Bacillus* sp. on changes in amino nitrogen, free sugar, thiamin, riboflavin, inorganic phosphate content as well as sensory qualities of traditional idli, rice soybean idli (black gram replaced by soybeans), dhokla and khaman (Table 17.5). The increase in thiamin and riboflavin contents as a result of fermentation is particularly notable in light of inadequacies of these vitamins in the diets of some Indian children. Bacteria may also play a role in the break down of phytate present in black gram. It was reported that a strain of *Leuconostoc mesenteroides* isolated from soybean idli secretes β -N-acetylglucosaminidase and α -D-mannosidase which are involved in the hydrolysis of herr agglutinin.

B. Waries

Waries (Panjabi waries) are a spicy condiment shaped in the form of a ball about 3 to 8 cm in diameter which are used in cooking with vegetables, grain legumes or rice in India dehulled grain legumes are soaked in water ground into a coarse paste, and mixed with spices and a small amount of paste from a previous fermented batch. Some spices used include asafoetida, caraway, cardamon, cloves, fenugreek, ginger, red pepper, and salt. After fermenting as a mass for 4 to 10 days at ambient temperature, the paste is formed into balls and air-dried in the sun. The surface of the balls becomes sealed with a mucilaginous coating during the drying process, thus

entrapping gases produced by yeasts present inside Yeasts identified as contributing to fermentation of waries are *Candida* spp. and *Saccharomyces cerevisiae*. Waries can also be prepared from bengal gram and mung bean flours.

Table 17.5 : Changes in Physical and Chemical Composition of Some Indian Fermented Foods^a

Fermented Food	Increase in Vol. (%)	pH after Fermentation	Percentage of Unfermented Values					
			Amino Nitrogen	Free Sugar	Thia-min	Ribo-flavin	Niacin	Inorg. P
Rice-black gram idli	80	4.0	177	284	109	171	148	284
Rice-soybean idli	56	4.5	158	308	183	349	167	292
Dhokla	51	5.1	129	408	158	213	173	288
Khaman	55	5.4	219	676	136	300	190	548

^aExperimental fermentation: from LAKSHMI (1978)

C. Papadam

Papadam is similar to waries but does not contain fenugreek or ginger. These circular tortilla-like wafers are prepared from a mixture of black gram, paste and spice which have been fermented for 4 to 6 h. Yeasts responsible for fermentation are the same as those found in waries, Papadams are served roasted or deep-fat fried and consumed as a relish.

D. Dhokla

Dhokla is a steamed fermented food prepared from a mixture of wheat semolina and bengal gram (2:1 ratio) flours. The flours plus about 3% salt are made into a thick batter by adding water and then allowing to ferment for about 14 h. Chopped fenugreek leaves are added to the dough before steaming for 20 min in a pan with oil. The product is then cut into pieces and may be seasoned with cracked mustard seeds before eating as a condiment with other breakfast foods in India.

E. Khaman

Khaman is a stemmed fermented condiment prepared from Bengal gram flour in India. Dehulled seeds are washed, soaked for

4 h, ground with water (2:3, Bengal gram: water) and seasoned with salt before fermenting for 12 h. Like dhokla, the dough is then steamed and seasoned with mustard seeds before eating.

F. Kenima

Kenima is a fermented soybean product resembling Indonesian tempeh but prepared in Nepal. Sikkim and the Darjeeling districts of India Soybeans are soaked overnight, dehulled and cooked in water for 2 to 3 h. The cooled soybeans are inoculated by the addition of a portion from a fermented batch, wrapped in leaves and fermented at 22 to 23°C for 24 to 48 h. The microorganisms responsible for the mucilaginous end product known as kenima have not been identified. When deep-fat fried and salted, kenima has a nutlike flavor much like that of tempeh.

G. Jalebies

Jalebies are a spiral-shaped, deep- fat fried confectionery product made from fermented wheat flour. The fresh fried jalebies are dipped in sugar syrup before serving. They are consumed throughout India, Nepal, and Pakistan.

H. Kanji

Kanji is a fermented beer-like beverage common in households and marketplaces in India. North Indian Kanji is prepared from purple or occasionally orange cultivars of carrots, beets, species, a portion from a previous batch of kangji, and water. South Indian kanji is prepared in two steps, the first involving the preparation of torani (a fermented rice liquor) and the second involving the fermentation of torani and a mixture of vegetables, spices and water. *Hansenula anomala* var. *anomala* has been isolated from kanji collected in North India (Delhi) whereas *H. anomala*. *Candida guilliermondii*, *C. tropicalis*, and *Geotrichum candidum* have been isolated from South Indian kanji.

Fermented Foods of Africa

A. Dawadawa

Dawadawa is a fermented locust bean (*Parkia filicoidea*) product prepared and consumed largely in West Africa. It is also known as *ƙpalugu* by the Kusasis and Dagombas of Northern

Ghana, iru or daddowa in Nigeria, kinda in Sierra Leone and neteton in Gambia.

The yellow powdery pulp is removed from the dark brown to black seeds, and the seeds are boiled in water with the possible addition of potash until slightly soft. The beans are then stored overnight in earthenware, metallic pots or baskets to further soften the coat. On the following day, the black seed coats are removed by hand rubbing the seed, rubbing them against the walls of the storage vessel, or by gently pounding them with a wooden pestle and mortar. Sand or wood ash may be used as abrasive agents to aid in the removal. The swollen cotyledons are then washed in water, boiled for 30 min and deposited at a depth of about 10 cm in a tray, pot or basket. Alternatively, the cotyledons may be put into a hole in the ground for fermentation. Also, the yellow portion of the first pulp and wood ashes may be sprinkled over the cotyledons at this time.

The preparation is covered with leaves or sheets of polyethylene and left to ferment for 2 to 3 days at ambient temperature. The microorganisms responsible for fermentation have not been determined but undoubtedly include sporeforming bacilli, lactic acid bacteria and probably yeasts. Metabolic activities of the microflora result in a mucilaginous, strongly proteolytic, ammoniacal smelling substance which covers and binds the individual beans. During fermentation, the beans change in color from light to dark brown and become greatly softened. Moisture is partially removed from the fermented mass by sun-drying before pounding into flattened cakes. The cakes may be further dried to prolong shelf: darkening during sun-drying is due to polyphenol oxidation.

The composition of unfermented locust bean and daddowa, a fermented locust bean product prepared in Northern Nigeria, is shown in Table 17.6.

B. Gari

A staple food prepared by fermenting the root of the cassave plant (also known as manioc, mandioca, apiun, yuca, cassada or tapioca in various parts of the world) is known as gari in the rain forest belt of West Africa. We have estimated that about 70% of the cassava grown in Nigeria is used for gari manufacturing. As described the traditional preparation of gari consists of the

following stages as illustrated in Fig. 17.10. First, the corky outer peel and the thick cortex are removed and the inner portion of the root is grated by hand on homemade rasps. The grated pulp is then packed into jute bags and weights are applied to express some of the juice. Fermentation takes place over a 3- to 4- day period at which time the cassava is sieved to remove coarse lumps and heated while constantly turning over a hot steel pan or in an oven. This process has been termed "garifying".

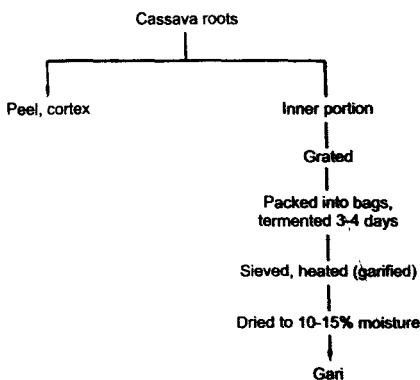


Fig. 17.10 : Flow diagram for preparing gari.

The moisture content of the fermented cassava is reduced to about 10 to 15% to yield a final product known as gari. In addition to water, gari contains 80 to 85% starch, 0.1% fat, 1 to 1.5% crude protein, and 1.5 to 2.5% crude fiber. Palm oil may be added to the product just before or after drying to give it color. We identified trace amounts of sodium manganese, iron, copper, boron, zinc, molybdenum, and aluminum in gari.

While it has been recognized for some time that fresh cassava roots contain cyanogenic glucosides, it is also known that these glucosides decompose during the traditional methods for preparing gari with the liberation of gaseous hydrocyanic acid. These researchers studied the microbiology of gari during its preparation and consistently isolated a bacterium (*Corynebacterium manihot*) and a fungus (*Geotrichaum candidum*) from grated cassava during the holding stage. They suggested that the process of detoxification of cassava root should be regarded as a two stage fermentation. In the first stage, *C.manihot* hydrolyzes the starch and produces various organic acids. This results in a decreased pH which causes spontaneous hydrolysis of cyanogenic glucosides with the liberation of gaseous hydrocyanic acid. The acid condition favors the growth of *G.candidum* which produces aldehydes and esters which gives gari its characteristic aroma and flavor. More recently attributed flavor development in gari to lactic acid bacteria, particularly *Lactobacillus* spp. and, to a lesser degree, *Streptococcus* spp.

Table 17.6 : Composition of Unfermented locust Beans and Daddawa, a Fermented Locust Bean Cake Product Prepared in Nigeria

<i>Component</i>	<i>Unfermented</i>	<i>Fermented</i>
Moisture	12.7	13.8
Crude protein	30.6	38.5
Total carbohydrate	49.1	23.6
Crude fiber	7.8	6.2
Ether extractable material	15.2	31.2
Ash	5.1	6.8
Minerals	250	5.50
Sodium	240	250
Zinc	15	18
Magnesium	80.5	83.3
Calcium	330	360
Copper	1.5	2.0
Iron	22.5	28.0
Phosphorus	280	320
Vitamins		
Thiamin	0.65	1.35
Riboflavin	0.45	1.30
Ascorbic acid	7.50	5.20
Toxic substances		
Oxalate	0.21	0.12
Hydrocyanic acid	0.0026	0.0012
Total phytic acid P	51.0	31.0
Phytic acid P as	15.0	7.5
% of total P		

C. Banku

A fermented starch-based Ghanaian food known as banku is prepared exclusively from maize or from a mixture of maize and cassava. The process involves steeping the raw material in water for 1 day, wet milling and fermenting for 3 days. The dough is then mixed with water (5:2, maize dough/water or 4:1:2, maize dough/cassava dough/water). Considerable stirring and kneading of the fermented dough is required to attain the correct consistency during subsequent cooking. The product is then put into a mold and served

Lactic acid bacteria and perhaps yeasts are responsible for fermentation.

D. Ogi

Maize is eaten in West Africa principally in the form of porridge known as ogi (Nigeria) or kenkey (Ghana): the Bantu (South African) equivalent to ogi is called mahewu. To prepare ogi, kernels of maize are soaked in warm water for 1 to 3 days, after which they are wet-milled and sieved with water through a screen mesh to remove fiber, hulls and much of the germ. The filtrate is fermented to yield a sour, white, starchy sediment known as ogi which is marketed as a wet cake wrapped in leaves. It may be diluted in water to 8 to 10% solids and boiled into a pap cooked and turned into a stiff gel (eke) before eating. Ogi is an important traditional food for weaning babies and a major breakfast cereal for adults.

The fermentation of maize to prepare ogi proceeds naturally without the addition of inoculants or enzymes. Lactic acid bacteria were found to be mainly *Lactobacillus plantarum*: aerobic bacteria included *Corynebacterium* and *Aerobacter*: yeasts identified were *Candida myoderma*, *Saccharomyces cerevisiae*, and *Rhodotorula*, and molds consisted of *Cephalosporium*, *Fusarium*, *Aspergillus*, and *Penicillium* species. *L. plantarum* was responsible for production of lactic acid, the main flavor base of ogi, whereas an increase in riboflavin and niacin content was attributed to *Aerobacter* also thought to contribute to flavor development.

We evaluated high-lysine maize for its suitability as a substrate for producing ogi. It was concluded from studies using controlled inocula and fermentation conditions that this nutritionally improved maize variety could be sacrificing final product sensory qualities.

E. Injera

Injera is Amharic word for an Ethiopian bread made from teff, wheat, barley, corn, sorghum or a mixture of these grains. However, the grain of choice is teff. To prepare injera, teff flour and water are combined with irsho, a fermented yellow fluid saved from a previous batch. The resultant thin, watery paste is generally incubated for 1 to 3 days. A portion of the fermented paste is then mixed with a portion of the original fermented flour to yield a clean-

looking, thin injera. Thick injera (aflegna), popular in rural Ethiopia, is teff paste which has undergone only minimal fermentation (12 to 24 h) and is characterized by a sweet flavor and a reddish color. A third type of injera (komtata) is made from over-fermented paste and, consequently, has a sour taste, probably due to proliferation of lactic acid bacteria. While the microflora responsible for fermentation of the sweeter type of injera have not been fully determined, yeasts probably play the greatest role. *Candida guilliermondii* apparently is a primary yeast in this process.

F. Kaffir Beer

Strictly speaking, kaffir beer is an African beverage made from kaffir corn, i. e., sorghum, a principal grain in some parts of the continent. However, similar beverages are prepared from other grains and starchy plant materials such as plantains and cassava.

Cereal grain is pounded using a wooden mortar and pestle, or by rubbing it between stones. Malt is prepared by soaking whole grain in water for 1 or 2 days after which it is sundried and allowed to mature before grinding. The pounded grain is made into a thin gruel, boiled, mixed with a small portion of uncooked malt and allowed to set overnight. On the second day, the mixture is boiled, cooled and allowed to stand until the fourth day when more pounded, boiled malted grain is added to the brew. On the fifth day the brew is strained through coarse baskets to remove some of the husks; the filtrate is now ready for consumption.

During the early stage of fermentation saccharification of starch occurs due to the growth of *Aspergillus flavus* and *Mucor rouxii*. The malt actually supplies little diastase to break down starch during the fermentation process.

G. Merissa

The process for preparing merissa beer is described. Sorghum grains are soaked in water for 1 day before spreading on the ground and covering with wet sacks or plant leaves. Germination proceeds for 2 days at which time the grains are sun-dried and milled into coarse flour. This represents the malting phase. Meanwhile, ungerminated sorghum is milled into flour and moistened with water. This dough undergoes fermentation for about 36 h and is then known as ajeen. The ajeen is cooked in a hollow steel container without further addition of water until it takes on a dark brown

color. The intermediate moisture product (Soorij) is extremely sour but has a pleasant caramelized flavor. An equal amount of water and about 5% of malt flour and 5% of good merissa are added to ferment for 4 to 5 h. At this stage, the product is called deboba.

Meanwhile, an amount of sorghum flour twice the size used for preparing the ajeen is divided into two equal lots: one lot is cooked to yield a greyish-brown paste and the other is cooked longer to produce a brown paste. The two are mixed and cooled to form a gelatinized material called futtara. Malt flour (5%) is mixed with the futtara which in turn is placed underneath the deboba surface as one solid mass. Fermentation (and liquefaction) proceeds for 8 to 10 h before the mass is strained to yield merissa (filtrate) and a residue (mushuk), which is used as animal feed. The principal microorganisms in merissa fermentation are lactic acid bacteria and a *Saccharomyces* spp. of yeast. Molds are not considered to play a role.

Other Fermented Products

A. Milk/Grain Products

Several indigenous fermented foods contain a mixture of animal milk and grain.

Kushik. To prepare kushik, dried parboiled whole wheat meal and yoghurt (1:1) are combined and allowed to ferment for 1 week. Curd from an equivalent volume of milk is added, and the mass is fermented for additional 4 to 5 days. The product is then sun-dried, ground into a powder and stored for later consumption. It is reconstituted to form a porridge to be eaten with pulses or bread, or cooked with chickpeas or green vegetables such as beet leaves.

Tarhana. Tarhana is also made from parboiled wheat meal and yoghurt, but in proportions of 2:1. Tomatoes, peppers, onions, garlic, salt, and spices are added and, after fermentation for several days, the product is dried. It is used largely for soup making.

Kishk. Kishk is a fermented product prepared from parboiled wheat and milk, and consumed in much of the Arab world. In Egypt, more milk than can be consumed is available at certain times of the year, so the surplus is stored in earthenware containers. Wheat is slowly boiled until it is soft, washed with water and dried. It is then coarsely ground and sieved to remove the seed coats.

In the meantime, laban zeer is prepared by concentrating salted sour milk. The powdered parboiled wheat is moistened with slightly salted boiling water and mixed laban zeer to obtain a homogeneous paste called hamma. After 1 day of fermentation, the hamma is kneaded; twice the volume of laban zeer added before it is diluted with water or milk is added to the hamma and left for another day. Spices such as pepper or cumin may also be added with the laban zeer. At this point, the mass is thoroughly mixed, formed into small balls and dried. The product is known as kishk. Lactic acid bacteria responsible for fermentation include *Lactobacillus plantarum*, *L. casei*, and *L. brevis*; yeasts also contribute to the process.

B. Kaanga-Kopuwai

The process of fermenting maize in water before eating is carried out in parts of New Zealand where the final product is termed kaanga-kopuwai (maize soaked in water) in Northland and kaanga-pirau (rotten corn) and kaanga-wai (water corn) among the Maoris of Central North Island and the Bay of plenty. The Maori process, is briefly summarized: mature whole maize cobs are placed unhusked in a jute sack and submerged in water, usually in streams, stagnant ponds or run-offs from pastures. The time required for proper fermentation is about 3 months, but the corn is said to stay fit for eating for an indefinite period if left in the water. If the sack begins to disintegrate, bundles of ferns are sometimes tied around it for preservation.

After 12 weeks of fermentation, the husks are yellow and soft and the kernels are full, but very soft and often slimy on the surface. Preparation for eating consists of scraping the kernels off the cob, removing the pericarp (optional), mincing, pulverizing and boiling with water to form a sort of gruel which is often eaten hot with sugar and milk or cream. The kernels may also be fried with salt and animal fat and eaten in this fashion.

Since the kaanga-kopuwai has been described as "rotten" maize by Westerners, it is safe to assume that considerable proteolysis occurs as a result of microbial activity during the fermentation process. An account of the microflora responsible for fermentation, however, has not been given.

C. Poi

Taro (*Colocasia esculenta*) corms are the principal material used to prepare poi in Hawaii and islands in the South Pacific. Corms are first cooked, peeled and ground or pounded to a fine consistency. The addition of water at this point results in fresh poi. Based on consistency, poi is designated as "one finger," "two finger" or "three finger," depending upon the number of fingers required for a satisfying mouthful. The second phase of poi preparation involves fermentation at ambient temperature for 1 to 3 days or longer. As fermentation progresses, texture changes from a sticky mass to one having a more watery and fluffy consistency. Poi is marketed commercially in glass jars and plastic bags.

Lactic acid bacteria are the predominant microflora during early stages of fermentation. *Lactobacillus delbrueckii*, *L. pastorianus*, *L. pentacetius*, *Streptococcus lactis*, and *S. kefir* produce large amounts of lactic acid and moderate amounts of acetic, propionic, succinic, and formic acids. *Candida vini* and *Geotrichum candidum* are prevalent in latter stages of fermentation. These fungi are thought to be responsible for imparting the pleasant fruity aroma and flavor to older poi.

D. Chicha

The word chicha is mainly applied to fermented maize chicha in Peru; however, it may also be prepared from fruits, cassava or mesquite. Over the centuries chicha has played a predominant role in religious and primitive fertility rites. The derivation of the word chicha describes the principal way in which it was made in the past, i.e., using saliva to convert starches to sugars to facilitate fermentation and increase the alcohol content.

Methods for preparing chicha in various parts of Peru differ. Good quality Alazá maize is shelled and kernels are immersed in water in earthenware pots and placed in the ground. After 12 to 8 h, the swollen maize is removed from the pot and spread in layers 5 to 7 cm thick and kept wet over a period of about 3 days to promote germination. The optimum stage of germination is reached when the plumule is about 1 cm long and tastes sweet. The germinated maize is then piled into heaps and covered for a period of 2 days during which it is said to be "humeando" or smoking because of the increased temperature due to biochemical activity. At this point

the parched maize is sun dried for 2 to 5 days and broken pieces of roots and seedling shoots, collectively known as *jora*, are separated from the maize kernels, *Jora* is milled into *pachucho* which is mixed with water and boiled for 3 to 4 h, gradually cooled, boiled again for about 4 h, gradually cooled, boiled again for about 4 h, cooled, strained through a suitable cloth or wire screen into a special pot reserved for *chicha*-making and allowed to ferment for at least 1 day. Presumably this pot would carry an inoculum of lactic acid bacteria and yeasts necessary for the desired fermentation. So long as the brew remains sweet, it is not ready for drinking, but as soon as it turns slightly acidic it is said to possess the correct flavor.

E. Pozol

Pozol is a fermented maize dough that, diluted in water, is consumed raw as a beverage by the Indian and Mestizo populations in Southeastern Mexico.

Their method for making pozol does not differ much from that used by Mayan ancestors. White maize kernels (1.5 kg) are boiled for about 1 h in 2 liters of water to which a handful of lime powder has been added. When the kernels are swollen and their pericarps are easily peeled, they are cooled, rinsed with water and drained to get what is called *nixtamal*. The *nixtamal* is ground to a coarse dough, shaped into rolls about 6 cm wide and 12 cm long, wrapped in banana leaves and allowed to ferment at ambient temperature for 5 to 8 days.

F. Legume-Based Milk Products

Soybean milk is an aqueous extract of soybeans which is commercially marketed as a beverage in several Asian countries. One can also prepare peanut milk from peanuts. Both products are characterized by a distinct beany green flavor which is judged undesirable by some individuals, but

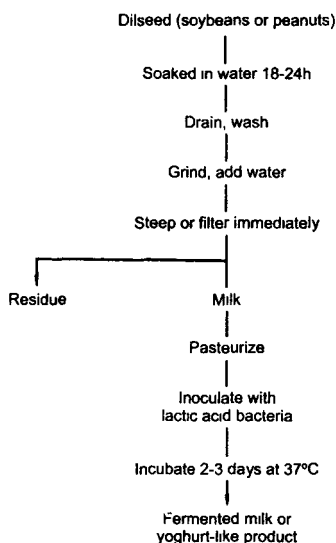


Fig. 17.11 : Flow diagram for preparing fermented oilseed milk products.

can be fermented with lactic acid bacteria to yield variously flavored and textured foods which have high sensory acceptability.

We evaluated growth rates of eight *Lactobacillus acidophilus* strains and four *Lactobacillus bulgaricus* strains in soybean milk enriched with glucose, lactose and sucrose. Almost all of the cultures could adapt themselves to the growth media tested. Taste panel evaluation of a chilled soybean beverage made using *L. acidophilus* revealed that the refreshing sour drink with a raisin flavor was highly acceptable to panel members. The authors were not able to suggest the origin of the raisin flavor and odor, nor was it concluded that the beany flavor of soybeans was merely masked by other flavors derived from fermentation or modified somehow. *L. brevis*, *L. cellobiosus*, *L. fermentum*, and *L. salivarius* subsp. *salivarius* and present in the soluble fraction of the cell.

The use of lysine-excreting mutants of *L. acidophilus* and *L. bulgaricus* to increase the lysine content of fermented soybean milk was demonstrated. The lysine content of soybean milk was increased by as much as 270% in a yoghurt-like product prepared by fermenting soybean milk with the mutants.

Fermentation of soybeans milk with *L. acidophilus* and subsequent flavoring, notably with lemon flavor, has been reported to yield an acceptable yoghurt-type product to achieve this success the soybean milk was supplemented with 5% sucrose and 2% Cheddar cheese whey solids. A higher amount of whey solids caused the milk to coagulate during sterilization. To prevent whey separation, 0.5 to 1.5% gelatin was added to the yoghurt product: the optimum level of gelatin depended upon the level of protein in the product. The authors reported that soybean yoghurt can be kept at 5°C for about 19 days without any significant change in acidity, pH and viable cell count.

We examined several extraction procedures for their suitability to yield desirable peanut milks for fermentation by four lactic acid bacteria. Studies revealed that a procedure in which peanuts were soaked in 1.0% sodium bicarbonate for 16 to 18 h, drained, washed with tap water, ground, steeped for 4 to 5 h in tap water, and filtered resulted in milk most desirable for fermentation (Fig. 17.11). The addition of lactose (2%) to pasteurized peanut milk before fermenting with *L. actobacillus bulgaricus* and *L. acidophilus* for 3 day at 37°C resulted in a custard-like product having 0.38 to 0.53%

titratable acidity at pH 4.76 to 4.43 respectively. Sensory panel evaluations of blended, fermented peanut milks containing added sucrose (2%) and fruit flavorings showed that the products were acceptable and competed favorably with flavored buttermilk. Fermented peanut milk substituted for buttermilk in a corn muffin recipe resulted in products with organoleptic characteristics not significantly different from those of the control. The authors expressed the need for further investigations for preparing fermented peanut milk products.

18

Lysine

Lack of the essential amino acid L-lysine in cereals triggered studies on L-lysine production by both chemical synthesis and microbial methods. Production of L-lysine on a large scale started, when a fermentation process using an auxotrophic mutant of *Corynebacterium glutamicum*. Recently another process using a regulatory mutant of *Brevibacterium flavum* was developed. Both processes have been further improved and are used for industrial production.

Biosynthetic Pathway of Lysine

Biosynthetically L-lysine is a member of the aspartate family (except in fungi) and the regulation of lysine synthesis has a close relationship with that of the other amino acids in the aspartate family. The aspartate family consists of aspartate, asparagine, methionine, threonine, lysine and isoleucine. The carbon skeletons of lysine and isoleucine are derived in part from pyruvate but are still considered part of the aspartate family. Isoleucine biosynthesis is best considered along with the valine pathway. Diaminopimelate, a lysine precursor, is needed for cell wall synthesis in bacteria but not for protein synthesis.

In *E. coli*, the formation of β -aspartyl phosphate (reaction 2, Figure 18.1) is catalyzed by three different aspartokinases, which have specifically evolved to fulfill the function of threonine (and isoleucine) formation, methionine formation and lysine formation, respectively. Although each aspartokinase would appear to be adapted for synthesis of β -aspartyl phosphate in an amount required for the specific end product, there appears to be no channelling of product and function in such a way. Rather, there appears to be a common pool of β -aspartyl phosphate from which all products are derived, as is demonstrated by the fact that the

predominant aspartokinase varies from one strain to another. Furthermore, the loss of one or even two of the three aspartokinase activities does not lead to auxotrophy, *i.e.* a deficiency in activity by any one aspartokinase is compensated for by derepression of one or both of the remaining isozymes.

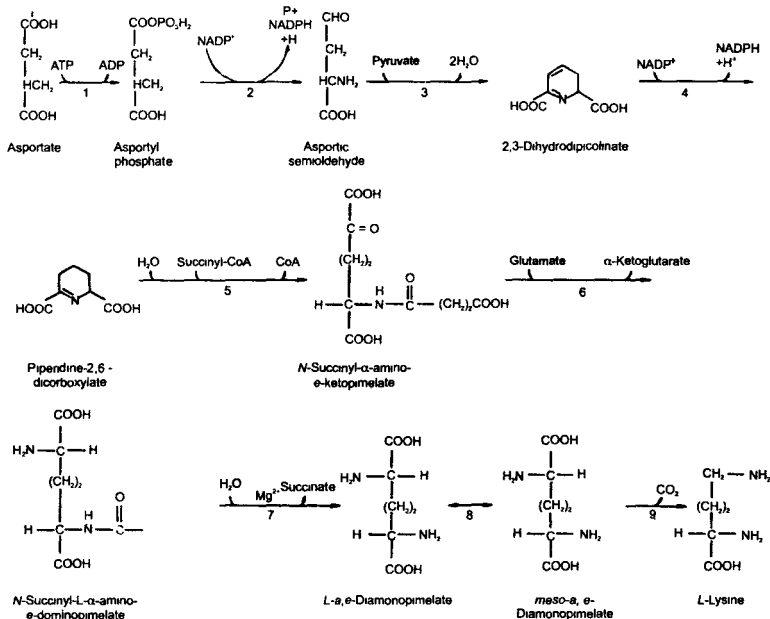


Fig. 18.1 : Pathway of lysine biosynthesis in bacteria (diaminopimelic acid pathway). Trivial names of enzymes: (1) β -aspartokinase, (2) aspartic β -semialdehyde dehydrogenase, (3) dihydrodipicolinate synthetase, (4) dihydrodipicolinate reductase, (5) N-succinyl- ϵ -keto-1- α -aminopimelate acid synthetase, (6) N-succinyl-L- α -aminodiaminopimelate transaminase, (7) N-succinyl-L- α -aminodiaminopimelate deacylase, (8) diaminopimelate epimerase, (9) diaminopimelate decarboxylase

Aspartokinase I activity is associated with a second activity, homoserine dehydrogenase I, carried on a bifunctional enzyme. Both activities are inhibited by threonine, but to different extents. The inhibition of aspartokinase activity is competitive with both substrates, whereas that of homoserine dehydrogenase activity is not. In *E.coli* K12 and presumably *Salmonella typhimurium*, aspartokinase II is also associated with homoserine dehydrogenase

activity in a bifunctional protein. The third aspartokinase in the Enterobacteriaceae appears not to be associated with any additional activities.

The pattern of multiple enzymes found in the Enterobacteriaceae for the multifunctional step, β -aspartyl phosphate formation, is not the only way the reaction is controlled. In *Bacillus polymyxa*, *Rhodopseudomonas capsulata* and the pseudomonas, there are only single aspartokinases, and their regulation is achieved by either a multivalent or a synergistic inhibition involving both lysine and threonine. In those organisms in which a single aspartokinases homoserine dehydrogenase activity is found on a separate protein. In general, homoserine dehydrogenase is inhibited by threonine but to various extents, so that there may be among these organisms some that contain two homoserine dehydrogenases. The remaining enzyme in the common pathway, β -aspartic semialdehyde dehydrogenase, catalyzes the NADPH-dependent reduction of B-aspartyl phosphate (reaction 2, Fig. 18.1).

With the exception of fungi in which lysine is formed by the pathway through α -aminoadipic acid, the biosynthesis of lysine occurs as a branch of the aspartate family of amino acids. The condensation of aspartic semialdehyde with pyruvate (reaction 3) yields a compound that spontaneously cyclizes and is then reduced in an NADPH-linked reaction (reaction 4). Ring opening for subsequent reaction is achieved by 'trapping' the open chain form by succinylation as in *E. coli* or by acetylation as in bacilli (reaction 5). After a transamination reaction (reaction 6), the acyl group is removed to yield L,L-diaminopimelate (reaction 7). Either this form or the *meso* form obtained by a specific recemase (reaction 8) or both (depending upon the organism) is used as a constituent of bacterial cell wall synthesis. The *meso* form is the substrate for the decarboxylase that catalyzes the final step in lysine synthesis (reaction 9).

Lysine Production with Homoserine Auxotrophs

A microbial process for L-lysine production was first developed by a combination of diaminopimelate production by a lysine-requiring auxotroph or a lysine-histidine-requiring double auxotroph of *E.coli* and decarboxylation of the compound by *Aerobacter aerogenes* or wild-type *E. Coli*. The yield of diaminopimelic

acid reached 24 g l^{-1} . Direct production of L-lysine from carbohydrate was developed first with a homoserine- or threonine- plus methionine-requiring auxotroph of *C. glutamicum* by the present author. The same type of process was reported with a homoserine-requiring auxotroph of *B. flavum*. The leaky homoserine-requiring auxotroph was recognized as a threonine-sensitive mutant because growth was inhibited by excessive threonine and the inhibition was released by addition of methionine. This phenomenon is due to feedback inhibition of residual homoserine dehydrogenase by threonine. Homoserine- (or threonine plus methionine)- requiring auxotrophs of other bacteria were also found to produce L-lysine, but the yields were lower than that from the homoserine-requiring auxotroph of coryneform bacteria.

Threonine auxotrophs and leucine auxotrophs of *C. glutamicum* produce fairly large amounts of L-lysine, but they are inferior to the homoserine auxotroph.

Cane molasses is now generally used as a carbon source in the industrial production of lysine, though other carbohydrate materials, acetic acid and ethanol can be used. The pH value of the medium is maintained near neutrality during the fermentation by feeding ammonia or urea. Ammonia and ammonium salts are generally good nitrogen sources, and urea can be used for organisms having urease activity. An example of a fermentation using cane molasses is as follows. The medium for first seed culture contained 2% glucose, 1% peptone, 0.5% meat extract and 0.25% NaCl in tap water. For the second seed culture, the medium contained 5% cane molasses, 2% $(\text{NH}_4)_2\text{SO}_4$, 5% corn-steep liquor and 1% CaCO_3 in tap water. The fermentation medium contained 20% cane molasses as glucose and 1.8% soybean meal hydrolyzate (as weight of meal before hydrolysis with 6 N H_2SO_4 and neutralization with ammonia water) in tap water. The fermentation (i) Medium contained 20% cane molasses as glucose and 1.8% soybean meal hydrolyzate (as weight of meal before hydrolysis with 6N H_2SO_4 and neutralization with ammonia water) in tap water. The fermentation was carried out at 28°C . Fig. 18.2 shows the time course of the fermentation using *C. glutamicum* No. 901 (a homoserine-requiring auxotroph), which produced 44 g of L-lysine (ii) Per litre in 60 h (The amount of L-Lysine was expressed as the weight of L-lysine monohydrochloride because the hydrochloride is the common form for use.) Foaming in the aerated culture can be

repressed by addition of proper antifoaming agents. The amount of the growth factors (homoserine or threonine and methionine) should be appropriate for the production of L-lysine. These are supplied in limited amounts and are suboptimal for the growth. The biotin concentration in the medium must generally be greater than $30 \mu\text{g l}^{-1}$. Cane molasses usually supplies enough biotin, but when beet molasses or starch hydrolysate is used, biotin must be added. Yields of L-lysine as the monohydrochloride reach 30–40% in relation to the initial sugar concentration.

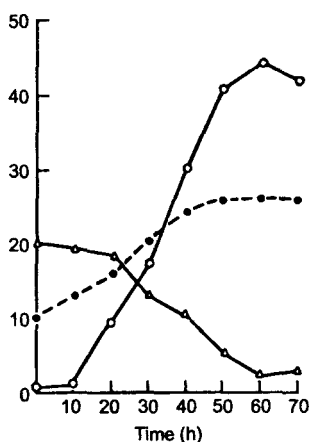


Fig. 18.2 : Time course of lysine fermentation; O, l-lysine (gl^{-1}); Δ , residual sugar (%), •, dry cell weight (gl^{-1})

Coryneform glutamic acid-producing bacteria can utilize acetic acid as a carbon source for growth and lysine production. L-Lysine production from acetic acid by a homoserine-leaky (threonine-sensitive) threonine auxotroph mutant of *Brevibacterium flavum* reached 75 gl^{-1} (as monohydrochloride) or 29% on the basis of acetic acid and glucose supplied. The medium contained 0.7% acetic acid, 0.2% KH_2PO_4 , 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 3.5% hydrolysate of soybean protein, 3.0% glycose, $50 \mu\text{g l}^{-1}$ biotin, and $40 \mu\text{g l}^{-1}$ thiamin HCl (pH 6.0). Fermentation was carried out at 33°C with feeding of a solution of acetic acid. The feeding solution contained 60% acetic acid composed of a mixture of acetic acid and ammonium acetate having

a molar ratio of 4:1 and 3% glucose. Feeding was controlled automatically until the end of the fermentation, keeping the pH value of the medium at 7.4. Some patents have been issued for a process to produce L-lysine from *n*-paraffins.

L-Lysine in fermentation both is recovered by adsorption on a cation exchange resin, and elution of the adsorbed lysine with dilute alkali. The eluate is neutralized with hydrochloric acid and crystals of L-lysine monohydrochloride are obtained by concentration of the eluate.

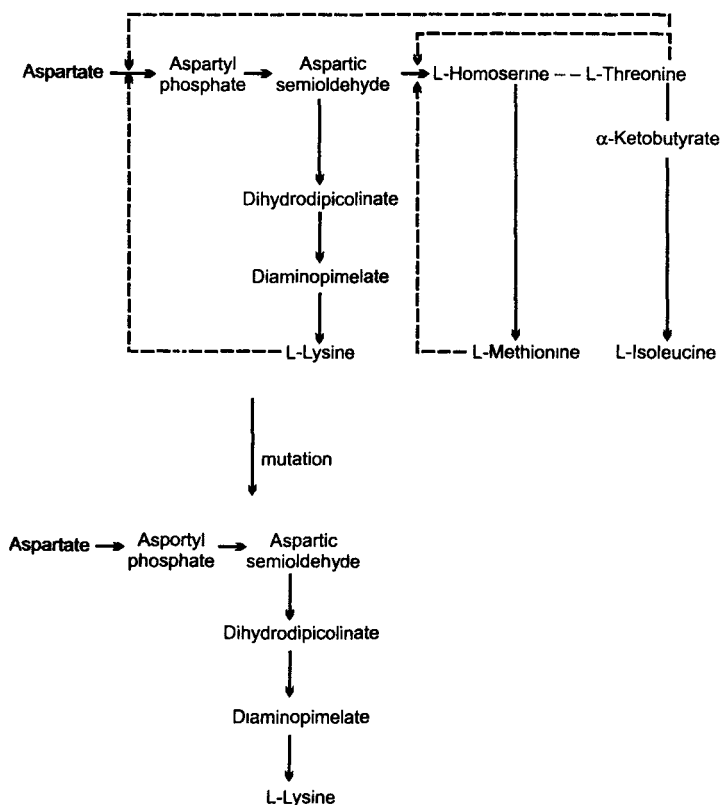


Fig. 18.3 : Deregulation of lysine biosynthesis in homoserine auxotroph of *Corynebacterium glutamicum*; ---, feedback inhibition; ---, repression

Deregulation in Lysine Overproducing Mutants

The mechanism of regulation of lysine biosynthesis in *C. glutamicum* is shown in Fig. 18.3. A similar regulatory pattern was also observed in *B. flavum*. Generally the reaction yielding the initial product on a biosynthetic pathway is subject to end product inhibition. Lysine inhibition of the aspartic semialdehyde-pyruvate condensation, the branch point to lysine biosynthesis, has in fact been demonstrated in *E. coli*, but in *C. glutamicum* neither lysine nor any other amino acid exerted an inhibitory effect even at concentrations of 10^{-2} M. Similar insensitivity of dihydrodipicolinate synthetase to lysine has been reported in *B. flavum*, *Bacillus cereus*, *Bacillus subtilis* and *Streptococcus faecalis*. Though homoserine dehydrogenase was inhibited by threonine and repressed by methionine in *C. glutamicum* and *B. flavum*, aspartic- β -semialdehyde dehydrogenase was not inhibited or repressed by any amino acid tested. Thus, the overproduction of lysine by homoserine or threonine auxotrophs can be accounted for by the release of their aspartokinases from concerted feedback inhibition, due to inability to produce threonine, and by the absence of any other major regulatory mechanism on the lysine biosynthetic pathway (Fig. 18.3). The blocking of homoserine synthesis at homoserine dehydrogenase results in the release of the concerted feedback inhibition by threonine and lysine on aspartokinase, and the aspartic semialdehyde produced proceeds to lysine through the lysine synthetic pathway on which no feedback inhibition is found, a situation which differs from that in *E. coli*. Lysine production by homoserine or threonine of *B. subtilis* may involve a similar mechanism, because similar metabolic control has been reported in *Bacillus species*.

A mutant of *E. coli* with aspartokinase insensitive to feedback inhibition by the end product but with normal catalytic activity has been isolated. Amino acid analogs have been used to select such regulatory, amino acid hyperproducing mutants in *E. coli*. The principle of this selective technique is simple: the analog acts as a pseudofeedback inhibitor, inhibiting the synthesis of the end product. The microorganism is unable to grow because it cannot replace the end product nutritionally. Deregulatory mutants are obtained as they are resistant to the analog and able to grow in its presence. A mutant of *B. flavum* resistant to 5-(β -aminoethyl)-L-cysteine (AEC), a lysine analog, produced fairly large amounts of

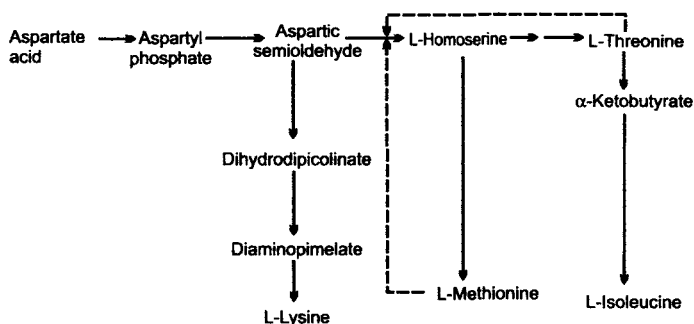


Fig. 18.4 : Deregulation of lysine biosynthesis in S-(β-aminoethyl) cysteine (AEC)-resistant mutant of *Brevibacterium flavum*;
 ---, feedback inhibition; ---, repression

L-lysine. Resistance to AEC brought about by the desensitization of aspartokinase also releases the concerted feedback inhibition. The conversion of aspartic semialdehyde to threonine is feedback inhibited by L-threonine. Thus the overproduced aspartic semialdehyde is channelled into L-lysine production (Fig. 18.4).

Production with Multiply Improved Mutants

Corynebacterium glutamicum

An increase in lysine yield (more than 10%) was obtained using a mutant of *C. glutamicum* which requires homoserine and leucine and is resistant to AEC. It produced 39.5 gL-lysine l⁻¹ in a medium containing 10% reducing sugars, expressed as invert (as cane molasses), while the homoserine plus leucine auxotroph produced 34.5 gL-lysine l⁻¹. As described earlier, leucine auxotrophs of *C. glutamicum* produced a fairly large amount of lysine. The results shown in Table 18.1 demonstrated the contribution of leucine auxotrophy and resistance to lysine, threonine and isoleucine analogs.

Table 18.1 : L-Lysine Production by some Auxotrophic Regulatory Mutants of *Coryne Bacterium glutamicum*

Strain	Genetic markers	L-Lysine yield(l ⁻¹)
ATCC21526	Homoserine ⁻ , leucine ⁻ , AEC ⁺	39.4
ATCC21523	Homoserine ⁻ , leucine ⁻ , AHV ⁺	38.2
ATCC21544	Homoserine ⁻ , leucine ⁻ , AMTB ⁺	38.1
ATCC21253	Homoserine ⁻ , leucine ⁻ ,	34.5
ATCC13032	Homoserine ⁻	28-30

C. glutamicum mutant, which requires homoserine, leucine and pantothenic acid and is resistant to AEC, produced 42 g L-lysine l⁻¹ in a cane molasses medium containing 10% reducing sugars expressed as invert.

Brevibacterium flavum

The prototrophic revertant 15-8 was derived from a citrate synthetase (CS) defective glutamate auxotroph of *B. flavum*. It showed low citrate synthetase activity and overproduced L-aspartic acid. The maximum production was 10.6 g l⁻¹ (about a 30% yield) when the strain was cultured in medium containing 36 g l⁻¹ of glucose for 48 h. Aspartate production by these mutants markedly depended on biotin concentration of the medium, as did the glutamate production by the original wild strain. When the revertant strain was cultured with excess biotin, acetic acid accumulated instead of aspartic acid. Therefore, the mechanism for the aspartate overproduction seems to be explained in the same way as that for the glutamate production.

A mutant resistant to AEC plus threonine derived from the revertant strain overproduced lysine with yields of 36%. This value is clearly higher than that obtained with an AEC-resistant strain which was directly derived from the wild strain. A homoserine auxotroph derived from the revertant strain produced 33 g L-lysine l⁻¹ (33 % yield), which was almost the same amount as that derived directly from the original wild strain.

Brevibacterium lactofermentum

As in *C. glutamicum*, leucine auxotrophy increased the lysine productivity of an AEC resistant mutant of *B. lactofermentum*. A leucine auxotroph derived from AEC resistant mutants produced 41 g L-lysine l⁻¹ while the parental strain produced about 18 g L-lysine l⁻¹. A kind of metabolic interlock was found in *B. lactofermentum*. Dihydrodipicolinate synthetase, an enzyme for the initial step of lysine biosynthesis, was repressed by L-lysine. This amino acid activated α -isopropylmalic acid synthetase, an initial enzyme for leucine biosynthesis, and also reversed the inhibition of the enzyme by leucine (Fig. 18.5). These facts explain the effect of leucine auxotrophy in a lysine overproducing mutant.

During the study of the effect of leucine on the accumulation of amino acids, it was noted that the accumulation of alanine increased

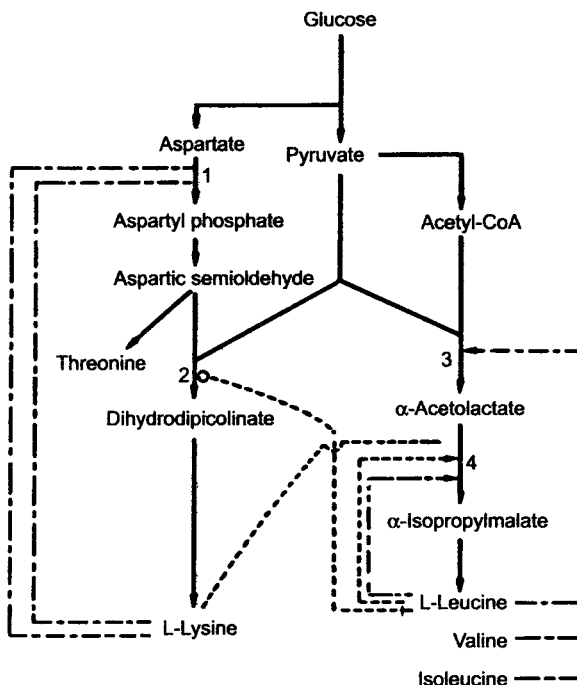


Fig. 18.5 : Mutual regulation in biosynthesis of lysine and leucine in *Brevibacterium lactofermentum*; trivial names of enzymes; (1) aspartokinase, (2) dihydropicolinate synthetase, (3) α -acetylacetyl-CoA synthetase, (4) α -isopropylmalic acid synthetase; ---, feedback inhibition; ---, repression; ----O, activation

as that of L-lysine decreased. Alanine can be formed from pyruvate by transamination, and directly from aspartate by aspartate β -decarboxylase (Fig. 18.6). But the activity of the aspartate β -decarboxylase was found to be approximately one tenth to one fiftieth of the transaminase activity. An alanine auxotroph was obtained by mutation from strain. The productivity of L-lysine was inversely proportional to the level of pyruvate L-amino acid transaminase. The best L-lysine producer, AJ 3799, yielded 39 g L-lysine l⁻¹ and lacked pyruvate-L-amino acid transaminase.

Thus the promotion of L-lysine production in *B. lactofermentum* by high concentrations of biotin is explained by the stimulation of pyruvate carboxylase by biotin which consequently leads to the increase of aspartate through the increase of oxaloacetate formation (Fig. 18.7).

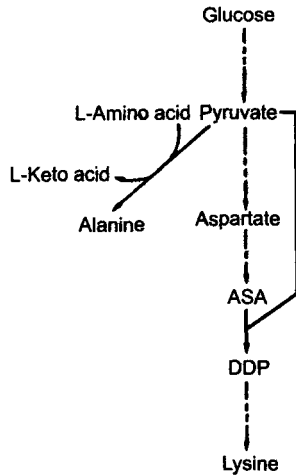


Fig. 18.6 : Pathway of lysine biosynthesis and alanine transaminase, ASA, aspartic β -semialdehyde; DDP, dihydrodipicolinate

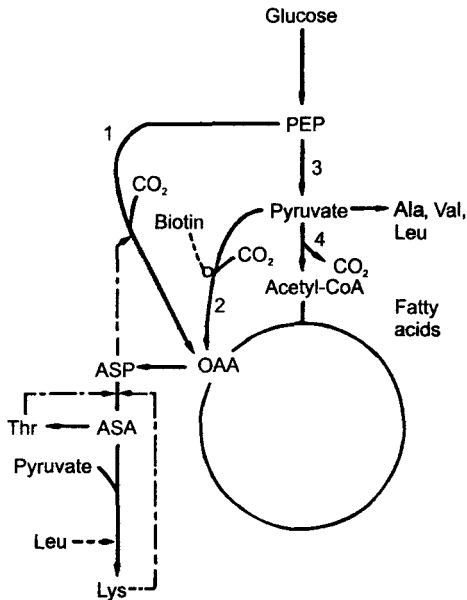


Fig. 18.7 : Pathway and regulation of lysine biosynthesis from glucose in *Brevibacterium lactofermentum*; ---, feedback inhibition; ----, repression; ---O, activation; trivial names of enzymes; (1) PEP carboxylase, (2) pyruvate carboxylase, (3) pyruvate kinase, (4) pyruvate dehydrogenase

Fluoropyruvate inhibited pyruvate dehydrogenase and increased lysine production of *B. lactofermentum* in the presence of high concentrations of biotin. Furthermore the mutant selected as fluoropyruvate-sensitive after mutagenization gave increased lysine production compared with the parental strain. The activity of its pyruvate dehydrogenase was found to be ~10-15% of the parental strain.

Aspartokinase of *B. lactofermentum* is subject to concerted feedback inhibition by lysine plus threonine as in *C. glutamicum* and *B. flavum*. A mutant resistant to AEC that grows in the presence of AEC plus threonine has been isolated. But α -chlorocaprolactam (CCL) or γ -methyllysine (ML) strongly inhibited the growth of *B. lactofermentum* by single addition, and the inhibition was reversed by lysine. The mutant isolated as a resistant to CCL or ML after mutagenic treatment produced 43 g L-lysine l⁻¹.

As a result of the breeding described above, *B. lactofermentum* has been selected and produces 48 g L-lysine l⁻¹ in a medium containing 10% glucose. The genealogy of the strain is shown in Fig. 18.8.

<i>Brevibacterium lactofermentum</i>	Lysine productivity (g l ⁻¹)
A ₁ 1511 (wild)	0
A ₁ 3445 (AEC ⁻)	16
A ₁ 3424 (AEC ⁻ , Alo ⁻)	33
A ₁ 3796 (AEC ⁻ , Alo ⁻ , CCL ⁻)	39
A ₁ 3991 (AEC ⁻ , Alo ⁻ , CCL ⁻ , ML ⁻)	43

Fig. 18.8 : Genealogy of lysine-producing mutants in *Brevibacterium lactofermentum*; AEC, S-(β -aminoethyl)-l-cysteine; CCL, α -chlorocaprolactam; ML, γ -methyllysine; FP, β -fluoropyruvate

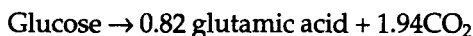
Aeration in Lysine Fermentation

The effect of oxygen tension on the production of L-lysine was studied employing the mutant of *B. lactofermentum*. Sufficient supply of oxygen to satisfy the cells, oxygen demand was essential for the maximum production of L-lysine. The dissolved oxygen level must be controlled at greater than 0.01 atm, and the optimum redox potential of culture media should be above -170 MV. An extremely oxygen deficient condition, when the degree of oxygen satisfaction represented by $rablK_RM$ (rab=cells' respiration rate, ml O₂ ml⁻¹;

$K_R M$ = maximum oxygen demand of cells, $\text{ml O}_2 \text{ ml}^{-1} \text{ min}^{-1}$) is less 0.3, led to the production of lactic acid at the expense of the lysine produced.

The production of L-lysine, L-threonine and L-isoleucine, which are biosynthesized *via* L- aspartic acid, decreased only slightly under insufficient oxygen supply.

An L-glutamic acid producing strain of *Brevibacterium* is generally able to convert α -ketoglutaric acid into succinic acid, and this lack of production is considered to favor the overproduction of glutamic acid. Two kinds of routes are considered for the biosynthesis of glutamic acid. One involves a glyoxylate cycle in which glutamic acid is biosynthesized by way of pyruvate, citrate and α -ketoglutarate. The other involves a PEP carboxylation system in which glutamic acid is formed by way of PEP, oxaloacetic acid, isocitric acid and α -ketoglutaric acid.



Judging from the amount of carbon dioxide evolved during the cultivation, the amino acid was produced from glucose employing both the glyoxylate cycle and the PEP carboxylation system. Biosynthesis of glutamic acid generates NAD(P)H_2 whether it is

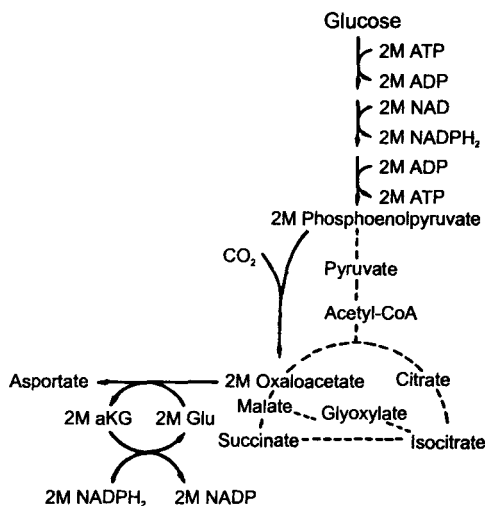


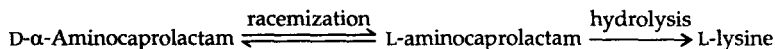
Fig. 18.9 : Metabolic pathway of aspartate biosynthesis involving carbon dioxide fixation (from Akashi *et al.*, 1979)

accomplished through the glyoxylate cycle or the PEP carboxylation system. This characteristic of glutamate biosynthesis was considered to cause marked inhibition of the product formation in oxygen-limited culture, because oxygen is required in amino acid fermentation mainly to reoxidize NADH_2 generated in the process of amino acid biosynthesis. Aspartic acid is also made through the glyoxylate cycle or the PEP carboxylation system. Biosynthesis of amino acid through the glyoxylate cycle is an NAD (P)H_2 generation process in marked contrast to that through the PEP carboxylation system which is an NAD (P)H_2 consuming one (Fig. 18.9).

In lysine fermentation, the degree of decrease in product formation in oxygen limitation was shown to be less significant than that in amino acid fermentations of the glutamic acid family. Biosynthesis of these amino acids of the aspartic acid family might be performed by way of PEP and oxaloacetate including carbon dioxide fixation in oxygen-limited culture.

Enzymatic Method

L-Lysine production from DL- α -aminocaprolactam was first studied using a fungus, *Aspergillus ustus*, which hydrolyzed only the L-form of α -aminocaprolactam. The remaining D-form was recycled after racemization. However, the yield was low. later, a more efficient process was developed for the conversion (Fukumura, 1976a, 1976b). Incubation of a mixture of 100 ml 10% DL- α -aminocaprolactam (adjusted to pH 8.0 with HCL), 0.1 g acetone-dried cells of *Cryptococcus laurentii* and 0.1 g acetone-dried cells of *Achromobacter obae* nov. sp. with gentle shaking at 40°C for 24 h resulted in the conversion of DL- α -aminocaprolactam to L-lysine with 99.8% yield.



C. laurentii produces L-aminocaprolactam hydrolase inductively in a medium containing L- α -aminocaprolactam, glucose and other ingredients. *A. obae* produces aminocaprolactam racemase using both D- and L- α -aminocaprolactam as an inducer. A similar optimal pH value of both enzymes allows the efficient conversion in what appears to be a single step.

Table 18.2 : Production Medium for Lysine Fermentation

Glucose	130 g l ⁻¹
KH ₂ PO ₄	1 g l ⁻¹
MgSO ₄ + 7H ₂ O	0.4 g l ⁻¹
FeSO ₄ + 7H ₂ O	0.01 g l ⁻¹
MnSO ₄ + 4H ₂ O	0.01 g l ⁻¹
(NH ₄) ₂ SO ₄	25 g l ⁻¹
DL-Alanine	0.35 g l ⁻¹
Biotin	50 µg l ⁻¹
Thiamine hydrochloride	200 µg l ⁻¹
Nicotinamide	0.5 mg l ⁻¹
Soybean hydrolysate ^a	15 mg l ⁻¹

^a 64g/liter of nitrogen is contained.

Lysine is produced by this enzymatic route by the Toray Company, Japan. Present capacity is 7500 ton yr⁻¹, increasing from 3000 to 4000 ton yr⁻¹. This process uses resting cells, and high grade L-lysine is obtained by carbon treatment and crystallization. The Toray process uses DL-aminocapro lactam, with an approximate conversion cost of 140–160 yen kg⁻¹, including purification. A schematic illustration of the enzymatic process is shown in Figure 18.10.

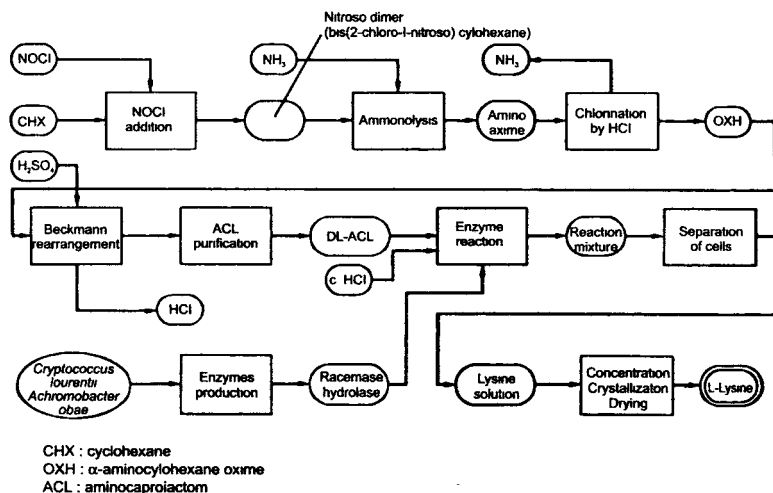


Fig. 18.10 : Process flow diagram for enzymatic L-lysine production

19 Tryptophan

L-Tryptophan was the first amino acid that was proved to be essential for human and animal nutrition, though it was later found that the requirement for it is only one half to one fifth as much as that for other essential amino acids. Although D-tryptophan is partly converted to the L-form *in vivo*, its nutritional value is much lower than that of the L-form.

At present, L-tryptophan is mainly used therapeutically, especially as a component of solutions for transfusion, but because it is an essential nutrient for humans and animals and various cereals such as corn are deficient in it, there is potential for its use as a food-and feed-supplement.

Biosynthesis of Tryptophan and Its Regulation

A general outline of the pathways consists of a 'common pathway' leading through shikimate to chorismate, after which there is branching to the specific pathways: to the three amino acids, to *p*-aminobenzoate, to menadione, to ubiquinone and to enterochelin (Fig. 19.1).

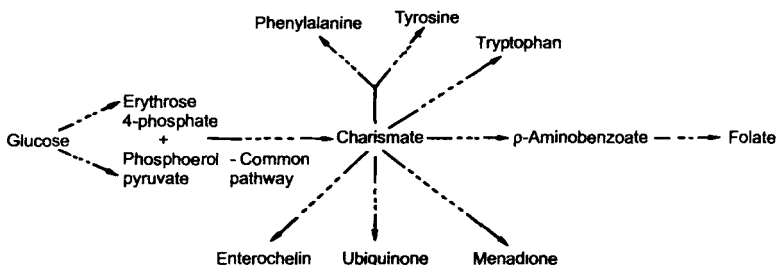


Fig. 19.1 : General outline of pathways for the formation of aromatic amino acids and vitamins in *Escherichia coli*

The Common Aromatic Pathway

Aromatic biosynthesis begins with the condensation of phosphoenolpyruvate and erythrose 4-phosphate to yield 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP; reaction i, Fig. 19.2). DAHP is cyclized upon the removal of phosphate to yield 5-dehydroquininate (reaction ii). The enzyme catalyzing this reaction requires NAD as a cofactor. Although there is no net oxidation or reduction in the overall reaction, there is an internal redox change; what had been carbon 7 of DAHP is reduced and what had been carbon 6 is oxidized (carbons 6 and 5-dehydroquininate). Removal of water and an NADPH-dependent reduction yield shikimate (reactions iii and iv). Shikimate is phosphorylated and condensed with another molecule of phosphoenolpyruvate (reactions v and vi). A second double bond is generated upon removal of the ring phosphate to yield the branch point compound, chorismate (reactions v to vii).

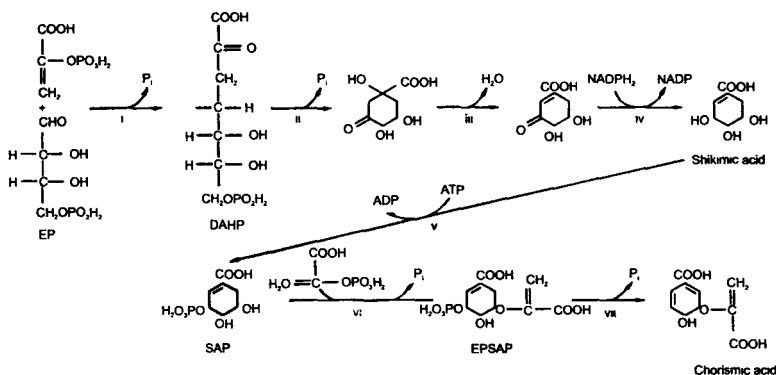


Fig. 19.2: Intermediates in the common pathway of aromatic biosynthesis, Abbreviations: PEP, phosphoenolpyruvate; EP, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHQ, 5-dehydroquininate; DHS, 5-dehydroshikimate; SAP, shikimate 5-phosphate; EPSAP, 3-enolpyruvylshikimate 5-phosphate, Trivial names of enzymes: i, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (DAHP synthetase); ii, 5-dehydroquininate synthetase; iii, dehydroquinase; iv, dehydroshikimate reductase; v, shikimate kinase; vi, 3-enolpyruvylshikimate-5-phosphate synthetase; vii, chorismate synthetase

In *Escherichia coli* and *Salmonella typhimurium* there are three DAHP synthetases: one is inhibited by phenylalanine, one by tyrosine and one by tryptophan. The same isozymic pattern has also been found in *Neurospora crassa*. In contrast to this isozymic pattern in *Bacillus subtilis* there is only a single DAHP synthetase activity and it is carried on the same protein that carries chorismate mutase. This protein (subunit A) forms a complex with another protein (subunit B) that has almost no activity by itself but which with subunit A exhibits shikimate kinase activity. Both shikimate kinase and DAHP synthetase activities are inhibited by chorismate and prephenate. That the inhibition may be due to binding of chorismate or prephenate to the active site of the mutase is suggested by the fact that proteolytic cleavage of the chorismate mutase fragment from the complex was accompanied by a loss of feedback sensitivity of DAHP synthetase.

In *Corynebacterium glutamicum* there is a strong synergistic inhibition of DAHP synthetase by phenylalanine and tyrosine. This inhibition was increased still further by the presence of exogenous tryptophan (nearly 90%). Still another pattern of inhibition found in organisms with single DAHP synthetase is that in which the enzyme is inhibited by only a single aromatic amino acid. In *Streptomyces aureofaciens* the enzyme is inhibited by tryptophan alone. The single enzyme in *pseudomonas aeruginosa* is somewhat similar in that tyrosine is the most effective inhibitor, although phenylpyruvate and tryptophan are also inhibitors.

Repression of enzyme formation in the common aromatic pathway has again been studied primarily in the Enterobacteriaceae. In *E.coli*, in addition to the tryptophan-specific repression of the tryptophan-sensitive DAHP synthetase and the tyrosine-specific repression of the tyrosine sensitive enzyme, there was a multiple repression of the phenylalanine-sensitive enzyme by phenylalanine plus tryptophan. The repression of the tryptophan and tyrosine-sensitive isozymes was dependent upon the *trp R* and *tyr R* loci, respectively. The *tyr R* gens specifies a repressor for the *tyr* regulon. The repression of the phenylalanine plus tryptophan-repressible isozyme also involves the participation of *tyr R* but not *trp R*. No *phe R* gene has been found in *E. coli*, but in *Styphimurium*, in which there is a *phe R* gene, it does not affect the multiply repressed, phenylalanine-sensitive isozyme. At least five of the other enzymes catalyzing the remaining steps of the common aromatic pathway are not end-product controlled.

The Tryptophan Pathway

The pathway is initiated by the conversion of chorismate to anthranilate in a glutamine-dependent reaction (reaction i. Fig. 19.3). The phosphoribosyl moiety of phosphoribosyl pyrophosphate is transferred to anthranilate (reactions ii). The indole ring is formed in two steps involving first an isomerization converting the ribose group to a ribulose and then a cyclization reaction to yield indoleglycerol phosphate (reactions iii and iv). The final reaction in the pathway is always catalyzed by a single enzyme which may contain either one or two types of subunit and consists of the cleavage of indoleglyceraldehyde 3-phosphate and condensation of the indole group with serine (reaction v).

The control of metabolite flow in the tryptophan pathway occurs by the inhibition of anthranilate synthetase by tryptophan. The enzyme has been extensively studied in *E. coli* and *S. typhimurium* with respect to both the structure of the protein and the interaction with substrates and inhibitors. The anthranilate synthetase of both organisms consists of two components. One, anthranilate synthetase component 1 (Col), can by itself catalyze the formation of anthranilate with ammonia as substrate. For the more efficient glutamine-dependent reaction, anthranilate synthetase component II (Coll) activity is also needed. Coll activity (amido transferase activity) resides in a portion of anthranilate phosphoribosyltransferase, the enzyme catalyzing reaction ii (Fig. 19.5). Thus, anthranilate is a complex of the two kinds of proteins. The coll activity in some other organisms is associated with other proteins. For example, in *N. crassa*, it is carried on the same protein that converts phosphoribosylanthranilate in two steps to indoleglycerol phosphate. In *B. subtilis*, *Serratia marcescens* and *Pseudomonas putida*, the amido transferase is carried on a separate protein apparently exhibiting no other activity. The anthranilate synthetase from *B. subtilis* could use only ammonia for the formation of anthranilate. Thus it appeared that anthranilate synthetase could actually function *in vivo* with ammonia.

Genetic studies on tryptophan biosynthesis in both *E. coli* and *S. typhimurium* have demonstrated a regulatory gene (*trp R*) that was thought to specify the repressor for the *trp* operon, and a *trp O* gene was thought to be the site of the repressor interaction. There was nearly a coordinate control over the levels of the five enzymes

except under extreme tryptophan starvation conditions and under conditions of full repression. A deviation from coordinate repression under repressing conditions also occurs and is attributed in both *E. coli* and *S. typhimurium* to the presence of a 'low level' promoter within the *trp D* gene of *E. coli* (*trp B* gene of *S. typhimurium*) that allows the unregulated expression of the last three genes in the operon.

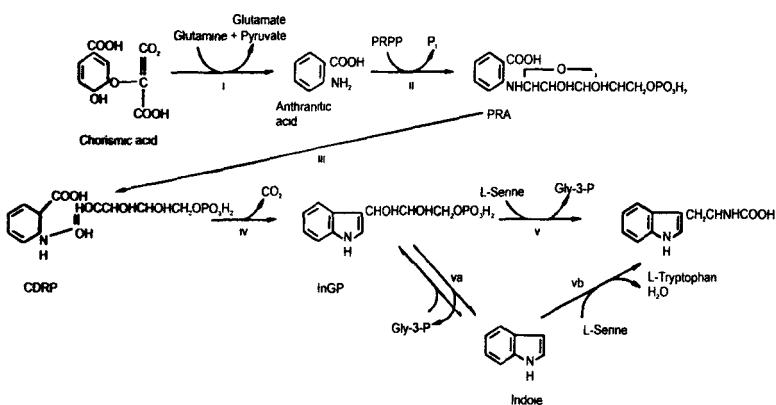


Fig. 19.3 : Intermediates in the tryptophan pathway, Abbreviations: PRPP, phosphoribosyl pyrophosphate; PRA, N⁵-phosphoribosyl-anthranilate; CDRP, 1-(O-carboxyphenylamino)-l-deoxyribulose 5-phosphate; InGP, indoleglycerol phosphate; Gly-3-P, glyceraldehyde 3-phosphate. Trivial names of enzymes: i, anthranilate synthetase; ii, anthranilate phosphoribosylpyrophosphate phosphoribosyltransferase; iii, N⁵-phosphoribosylanthranilate isomerase; iv, indole-3-glycerol-phosphate synthetase; v, (a or b), tryptophan synthetase (A or B)

Regulation of Tryptophan Biosynthesis in *Brevibacterium flavum*

Regulation of aromatic biosynthesis in *Brevibacterium flavum* is similar to that of *Corynebacterium glutamicum*. because of the industrial importance of these bacteria and of the more detailed studies of the former, the results with *B. flavum* will be described here.

In *B. flavum* DAHP synthetase of the common pathway is feedback-inhibited by only two of the three end products.

phenylalanine and tyrosine. Other amino acids and intermediate metabolites including tryptophan, chorismate and prephenate, were not inhibitory. Formation of DAHP synthetase was affected neither by tyrosine, phenylalanine and tryptophan alone nor by a combination of the three amino acids. In a phenylalanine auxotroph under phenylalanine-limiting conditions, overproduction of tyrosine strongly repressed the enzyme, while in a tyrosine auxotroph under tyrosine-limiting conditions, enzyme production was slightly derepressed. Tryptophan markedly reversed the repressive action of tyrosine.

The formation of DAHP synthetase was strongly repressed by overproduced tyrosine inside the cells under phenylalanine-limiting conditions, but formation of DAHP was only weakly repressed by addition of excess tyrosine to the culture medium, probably due to low permeability of the cells to tyrosine. It was also considerably derepressed under tyrosine-limiting conditions, therefore it seems that the DAHP synthetase of *B. flavum* is repressed by tyrosine but not by phenylalanine. Tyrosine at a normal level in wild-type cells seems to repress the enzyme only partially unless the tryptophan level is markedly reduced. Furthermore, the strong repressive action of overproduced tyrosine was also reversed by the addition of tryptophan. Thus, tryptophan does not repress enzyme formation but reverses the repressive action of tyrosine in *B. flavum*.

Chorismate mutase is composed of two inactive components (AB.) which are separated from each other by gel-filtration but associate to form an active complex in the presence of the substrate. Component A is a bifunctional enzyme and acts as DAHP synthetase.

Anthranilate synthetase from *Brevibacterium flavum* required chorismate, glutamine and Mg^{2+} for its activity. The enzyme was strongly and specifically inhibited by tryptophan, the metabolic end product. The inhibition by tryptophan was competitive with respect to chorismate and uncompetitive with respect to glutamine. In the presence of tryptophan, homotropic cooperativity of chorismate was observed. D-Tryptophan, phenylalanine, tyrosine, histidine and indole scarcely affected the enzyme activity.

Among the six tryptophan enzymes, two dissociable enzyme complexes were observed; one included tryptophan synthetase A

(TS-A) and tryptophan synthetase B(TS-B), the other *N*-5'-phosphoribosylanthranilate isomerase (reaction iii. from Fig. 19.3) and indole-3-glycerol-phosphate synthetase (reaction iv. Fig. 19.3). When tryptophan auxotrophs derived from *B. flavum* which lacked TS-A, TS-B or anthranilate phosphoribosylpyrophosphate phosphoribosyltransferase (reaction ii. Fig. 19.3) were cultured in media containing limiting or excess tryptophan, the specific activities of all six tryptophan enzymes were much higher under conditions of limiting tryptophan than under conditions of excess tryptophan. This indicates that the formation of all the tryptophan enzymes was repressed by tryptophan. However, the ratio of specific activities under the two conditions was not constant among these enzymes. When the rates of synthesis of the enzymes during tryptophan starvation were compared, those of anthranilate synthetase (reaction i. Fig. 19.3), *N*-5'-phosphoribosylanthranilate isomerase (reaction iii. Fig. 19.3). TS-A and TS-B were coordinate, while the specific activity of indole-3-glycerol-phosphate synthetase (reaction iv. Fig. 19.3) did not increase significantly.

Prephenate aminotransferase, the first enzyme of the tyrosine-specific branch, was not regulated at all by tyrosine nor by the other aromatic amino acids, while prephenate dehydratase, the enzyme for phenylalanine synthesis, was inhibited, but not repressed, by phenylalanine and activated by tyrosine. Moreover, this inhibition by phenylalanine was competitively recovered by the presence of tyrosine. The ratio of each biosynthetic flow for tyrosine and phenylalanine at the prephenate branch-point was calculated from the kinetic equations of prephenate aminotransferase and prephenate dehydratase, the first enzyme in the phenylalanine-specific branch. It showed that tyrosine was synthesized in preference to phenylalanine when phenylalanine and tyrosine were absent. Furthermore, this preferential synthesis was diverted to a balanced synthesis of phenylalanine and tyrosine through activation of prephenate dehydratase by the tyrosine thus synthesized. The feedback inhibition of prephenate dehydratase by phenylalanine was proposed to play a role in maintaining a balanced synthesis when supply of prephenate was decreased by feedback inhibition of DAHP synthetase, the common key enzyme. Overproduction of the end products in various regulatory mutants was also explained by these results. Fig. 19.4 summarizes the major regulatory mechanisms for aromatic amino acid biosynthesis in *B. flavum*.

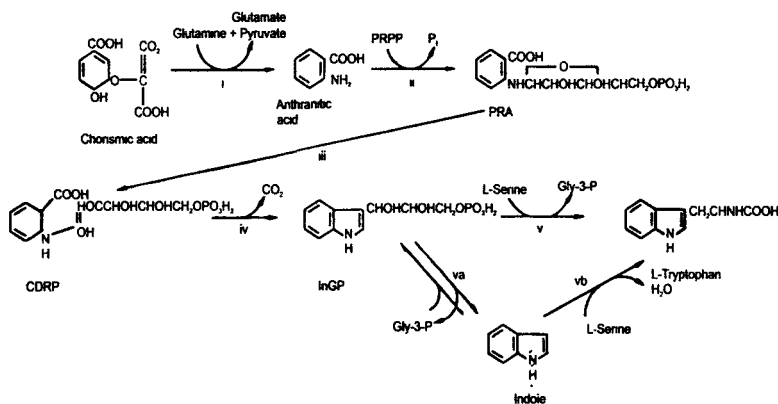


Fig. 19.4: Regulation of aromatic amino acid-biosynthesis in *Brevibacterium flavum*. Trivial names of enzymes and symbols: i. DAHP synthetase; ii. anthranilate synthetase; iii. chorismate mutase; iv. prephenate dehydratase; v. prephenate aminotransferase; vi. pretyrosine dehydrogenase; ---, feedback inhibition; ---, activation

Tryptophan Production by Fermentation

Corynebacterium glutamicum

Fig. 19.5 shows the genealogy of L-tryptophan-producing mutants of *Corynebacterium glutamicum*. Mutants producing a large amount of L-tryptophan were derived from a phenylalanine and tyrosine double auxotroph of *C. glutamicum* KY 9456 which produced only a trace amount of L-tryptophan and anthranilate. A mutant (4 MT-11), which, in a stepwise manner, acquired resistance to 5-methyltryptophan (5MT), tryptophan hydroxamate (TrpHx), 6-fluorotryptophan (6FT) and 4-methyltryptophan (4MT), produced L-tryptophan to a concentration of 4.9 g l⁻¹ in a cane molasses medium containing 10% reducing sugar as invert. L-Tryptophan production with this mutant was inhibited by L-phenylalanine and L-tyrosine. Accordingly, mutants resistant to phenylalanine and tyrosine analogs, such as *p*-fluorophenylalanine (PFP), *p*-aminophenylalanine (PAP), tyrosine hydroxamate (TyrHx) and phenylalanine hydroxamate (PheXx), were derived from this mutant. One of the mutants thus obtained (Px-115-97) produced 12 g l⁻¹ of L-tryptophan in the molasses medium. The medium used

	L-Tryptophan produced (g/l) ^a
KY9456 Phe, Tyr ↓ 5MT ^b , TrpHx ^c , 6FT ^d , 4MT ^e 4MT-11 ↓ PFP ^f PEP-2-32 ↓ PFP ^f PAP-126-50 ↓ TyrHx ^g Tx-49 ↓ PheHx ^h Px-115-97	0.15 4.9 5.7 7.1 10.0 12.0

Fig. 19.5 : Genealogy of L-tryptophan-producing mutants of *Corynebacterium glutamicum* and their L-tryptophan productivity. Medium used for production; cane molasses containing 10% reducing sugar as invert. Abbreviations: 5MT, 5-methyltryptophan; TrpHx, tryptophan hydroxamate; 6FT, 6-fluorotryptophan; 4MT, 4-methyltryptophan; PFP, *p*-fluorophenylalanine; PAP, *p*-aminophenylalanine; TyrHx, tyrosine hydroxamate; PheHx, phenylalanine hydroxamate.

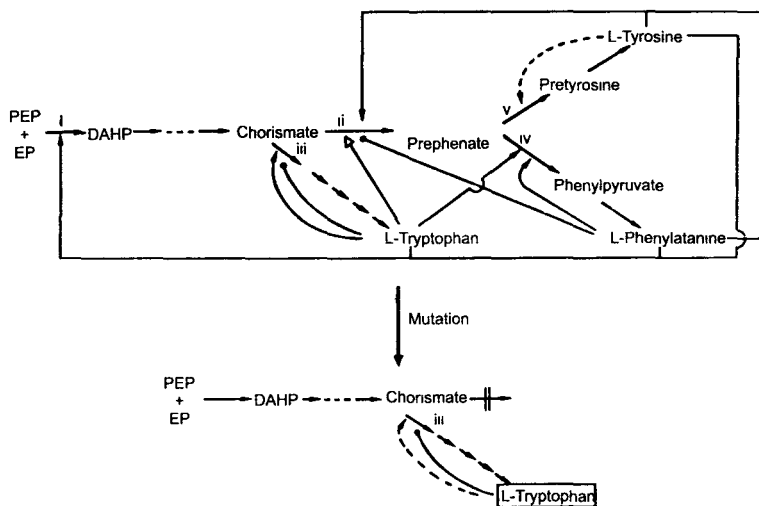


Fig. 19.6 : Regulation in aromatic amino acid biosynthesis in *Corynebacterium glutamicum* and deregulation in tryptophan producing mutant. Abbreviations: PEP, phosphoenolpyruvate; EP, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonic acid 7-phosphate, Trivial names of enzymes and symbols; i. DAHP synthetase; ii. chorismate mutase; iii. anthranilate synthetase; iv. prephenate dehydratase; v. pretyrosine dehydrogenase; —→, feedback inhibition;→, partial inhibition; —→, activation; □, overproduced metabolite; —→, blocked reaction; —●, repression.

had the following composition: 10% reducing sugars as invert (as cane molasses), 0.05% KH_2PO_4 , 0.05% K_2HPO_4 , 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2% $(\text{NH}_4)_2\text{SO}_4$, 1% corn-steep liquor and 2% CaCO_3 (pH 7.2). Production of L-tryptophan with the mutant was still sensitive to L-phenylalanine and L-tyrosine. Hence, further genetic improvement of the strain may be possible.

Mechanism of Overproduction of Aromatic Amino Acids

Regulatory properties of the enzyme involved in aromatic amino acid biosynthesis in *C. glutamicum* wild and mutant strains were investigated. The overall control pattern (Fig. 19.6) is a new addition to the list of control patterns in aromatic amino acid biosynthesis in microorganisms. A phenylalanine and tyrosine double auxotrophic L-tryptophan producer, Px-115-97, has anthranilate synthetase partially released from the inhibition by L-tryptophan and DAHP synthetase of a wild type. L-Tryptophan production by the mutant appeared to be caused by the release from the feedback inhibition of anthranilate synthetase by L-tryptophan and blockage of chorismate mutase.

Brevibacterium flavum

A 5-fluorotryptophan (5FT) resistant histidine auxotrophic mutant of *Brevibacterium flavum* produced 2.4 g l^{-1} of L-tryptophan. Production was increased to 3.8 g l^{-1} using a *m*-fluorophenylalanine (MFP) resistant mutant derived from the 5FT resistant one. A phenylalanine auxotrophic mutant from the latter mutant produced 6.2 g l^{-1} of L-tryptophan.

A 5-FT-resistant mutant, No. 187, derived from a tyrosine auxotrophic *p*-fluorophenylalanine (PFP) resistant mutant produced 8.0 g l^{-1} of L-tryptophan. Addition of resistance to a substrate analog of anthranilate synthetase, azaserine, gave higher tryptophan productivity. Thus an azaserine resistant mutant A-100 produced 10.3 g l^{-1} of L-tryptophan (Shiio *et al.*, 1982). The production increased to 11.4 g l^{-1} when L-serine was added. In the mutant anthranilate synthetase activity increased to a level twice that in the parent strain though it was inhibited in a similar manner to that of the parent. Furthermore, DAHP synthetase increased three-fold and was less sensitive to feedback inhibition by phenylalanine and tyrosine.

Production by Microbial Conversion

Scientists selected *Hansenula anomala* for microbial production of L-tryptophan using precursors such as anthranilic acid or indole. With *Hansenula anomala*, L-tryptophan production from anthranilic acid reached 5.7 g l^{-1} .

Afterwards, various microorganisms including auxotrophic and regulatory mutants were selected by different workers. They include *Candida fumicola*, *Corynebacterium glutamicum*, *Bacillus subtilis*, *Escherichia coli* and other species. By feeding anthranilic acid to a derepressed anthranilic acid auxotroph of *Bacillus subtilis*, 5.5 g l^{-1} of L-tryptophan were produced from 5 g l^{-1} of anthranilic acid. *Candida utilis* (synonym *Torulopsis utilis*) 295-t produced 6.4 g l^{-1} of L-tryptophan from 4.2 g l^{-1} of anthranilic acid in 36 h. By feeding indole, at 5-methyltryptophan-resistant mutant of *B. subtilis* ATCC 21336 produced 10.4 g l^{-1} of L-tryptophan in 96 h with a medium containing 7% glucose (Thieman and Pagani, 1972). A strain carrying an *F. Try* episome in addition to the chromosomal *try* operon was obtained by sexduction from a feedback-resistant and derepressed mutant of *E. coli* K12 which is resistant to 5-methyltryptophan (5MT) and 5-fluorotryptophan (5FT). This strain produced 5 g l^{-1} of L-tryptophan as a result of feeding with indole (1.5 g l^{-1}) and L-serine (7 g l^{-1} ; Sahm and Zahner, 1971).

A *Bacillus subtilis* mutant resistant to 5-fluorotryptophan produced 9.6 g l^{-1} of L-tryptophan in a medium containing 1% glucose and 5% soluble starch with continuous feeding of anthranilic acid. A further improved mutant which is resistant to 5-fluorotryptophan and 8-azaguanine produced 15.6 g l^{-1} of L-tryptophan in a medium containing 10% glucose with feeding of anthranilate solution. Yield of L-tryptophan compared with consumed glucose was 17.4% and conversion of anthranilate into L-tryptophan was 99%.

Recombinant DNA techniques were applied for enhancement of the tryptophan productivity of *E. coli*. A multiple mutant (*trp R*, *traA*) of *E. coli* was transformed with a recombinant plasmid containing a feedback resistant *trp* operon. The transformed strain produced 5.5 g l^{-1} of L-tryptophan by culturing for 24 h in a medium containing 5% glucose with feeding of anthranilic acid.

Production by Enzymatic Methods

Tryptophanase, which catalyzes synthesis of L-tryptophan by reversal of the α, β -elimination reaction at rates similar to the forward reaction, was utilized for production of L-tryptophan and related compounds such as 5-hydroxytryptophan. The culture broth of *Proteus rettgeri* (AJ 2770) was used as the enzyme for the reaction. For the synthesis of L-tryptophan, a reaction mixture contained 6.0 g of indole in 10 ml of methanol, 8.0 g of sodium pyruvate, 8.0 g of ammonium acetate, 0.001 g of pyridoxal phosphate, 0.1 g of Na_2SO_4 and 100 ml of the cultured broth in a total volume of 120 ml. After the pH value of the mixture was adjusted to 8.8 with 6 N KOH, it was incubated at 34°C for 48 h. Under these conditions, 7.5 g of L-tryptophan were synthesized. Similarly, 5-hydroxy-L-tryptophan was synthesized from 5-hydroxyindole, pyruvate and ammonia.



When the produced L-tryptophan was removed from the reaction system by precipitation with inosine, 83.3 g l^{-1} of L-tryptophan were produced. The molar conversion yield was 96%. More recently *Achromobacter liquidum* was used for L-tryptophan production from indole and L-serine. High enzyme activity was obtained by growing in a medium containing 0.2% L-tryptophan and 0.5% L-glutamate in addition to other ingredients. The cells thus obtained produced 96.6 g l^{-1} of L-tryptophan after 3 days' reaction with a mixture containing indole 60 g l^{-1} , L-serine 60 g l^{-1} , pyridoxal phosphate 0.5 mM and potassium phosphate buffer 0.1 M (pH 9.0). Crystals of L-tryptophan separated out.

Tryptophan synthetase of *E. coli* was utilized for L-tryptophan production from indole and L-serine or DL-serine. When the cells of *Pseudomonas putida* were added to the reaction mixture to racemize serine, 23.5 g l^{-1} of L-tryptophan were produced and conversion of DL-serine into L-tryptophan reached 81%.

Flavobacterium aminogenes nov. sp. has an intracellular enzyme system which degrades aromatic amino acid hydantoins into corresponding L-amino acids. With intact cells, 50 g l^{-1} as wet base, 10 g l^{-1} of DL-5 indolylmethylhydantoin were consumed and 7.4 g l^{-1} of L-tryptophan (a molar yield of 82%) were produced after 35 h incubation. Molar conversion of DL-tryptophanhydantoin into L-tryptophan was 100% when 5% each of DL-tryptophanhydantoin and inosine were treated at 40°C for 100 h.

with cells of a mutant of *F.aminogenes* in which the activity to break down tryptophan had been reduced. In this system inosine removes L-tryptophan from the reaction system by forming a complex with it. Spontaneous racemization of the substrate allowed the conversion of the D-form of hydantoin into L-tryptophan. The DL-5-indolymethylhydantoin-hydrolyzing enzymes were found to be inducible and intracellular (Fig. 19. 7).

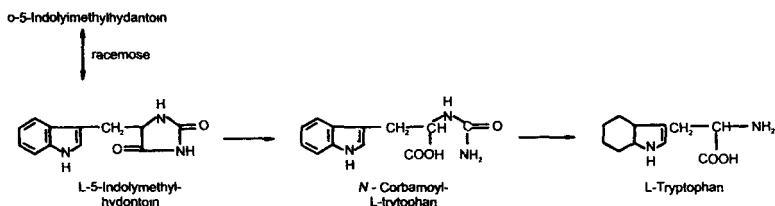


Fig. 19.7 : Possible scheme of L-tryptophan production from indolymethylhydantoin

Recovery

L-Tryptophan in the culture broth is recovered conveniently using ion exchange resin. For example, strongly acidic cation exchange resins (H^+ form) adsorb the L-tryptophan in the culture supernatant. After washing, the resin is subjected to elution with 0.5 N aqueous ammonia and then the resulting eluate is concentrated to obtain crude crystals of L-tryptophan. The crude crystals are dissolved in a small amount of hot 50% aqueous ethanol. The resulting solution is decolorized with active carbon and cooled, whereby L-tryptophan is recrystallized.

Non-ionic synthetic polymers like Permutit DR can adsorb aromatic amino acids such as tryptophan, phenylalanine and tyrosine. These characteristics are applied for the selective adsorption of aromatic amino acids from mixtures of amino acids.

20

Aspartic Acid

Aspartic acid was first obtained by hydrolysis of asparagine isolated from the juice of asparagus and its chemical structure was confirmed by chemical synthesis.

The amino acid has received considerable attention as a medicine since the report on its physiological and therapeutic importance. At present, it is widely used, not only in medicines but also as a food additive.

Recently Aspartame[®], a dipeptide of L-aspartic acid and L-phenylalanine methyl ester, was commercialized as a low calorie sweetener. Thus, demand for L-aspartic acid is expected to increase rapidly as it is a raw material for the synthesis of the dipeptide.

Production of L-Aspartic Acid

The methods for industrial production of L-aspartic acid can be classified into two categories: fermentative and enzymatic.

Fermentative Production

In practice, industrial production of L-aspartic acid has been carried out by the fermentation method using fumaric acid.

Use of glucose as a main carbon source

One of the several patents in this area describes a glutamate auxotroph strain derived from *Brevinacterium flavum*. When this strain was cultured in a nutrient medium containing 3.6% glucose and 0.5% L-glutamic acid at 30°C for 4 days, the final concentration of L-aspartic acid in the medium was 4 g l⁻¹.

A prototrophic revertant derived from a citrate synthase-defective glutamate auxotroph of *Brevibacterium flavum* has been employed for the overproduction of L-aspartic acid. The revertant

showed low citrate synthase activity and overproduced L-aspartic acid. When the revertant was cultured in a nutrient medium containing 36 g l^{-1} of glucose at 30°C for 48 h the maximum production was 10.6 g l^{-1} corresponding to a yield of approximately 30% from glucose.

Conversion of fumaric acid to aspartic acid by fermentation has been investigated in mixed cultures of *Rhizopus* sp., producing fumaric acid from glucose, and *Proteus vulgaris* having high aspartase activity. In the fermentation, the yield of L-aspartic acid was found to depend on the amount of fumaric acid accumulated in the initial fermentation process.

Use of fumaric acid as a main carbon source

In the course of screening for amino acid-producing microorganisms, a number of them belonging to *Bacillus* sp. had the ability to form L-aspartic acid from fumaric acid. Among the microorganisms tested, a strain of *Bacillus megaterium* was chosen as the most suitable for production of L-aspartic acid, and its cultural conditions for the formation of L-aspartic acid from fumaric acid were investigated. The strain was precultured in a medium containing 0.5% fumaric acid for 24 h and a fumaric solution neutralized with ammonia was then added to the fermentative broth. When the broth was further incubated for 72 h, L-aspartic acid was accumulated at the level of 10.6 g l^{-1} and obtained at near 80% yield from fumaric acid.

Scientists screened a number of microorganisms accumulating L-aspartic acid from fumaric acid and ammonium salts, and selected *Pseudomonas fluorescens* 6009-2 and a mutant, Ki-1023, derived from *Escherichia coli* K-12 as suitable strains for production of L-aspartic acid. In the case of *P. fluorescens*, fumaric acid was completely consumed in 3 days, and conversion of fumaric acid to L-aspartic acid was greater than 95% on the partially stationary culture, i.e. stationary culture after shaking for one day. In the case of *E. coli*, fumaric acid was completely consumed in 3 days and the maximum conversion of fumaric acid to L-aspartic acid was attained in 4 days. The maximum yield of L-aspartic acid almost reached the theoretical value of 56 g l^{-1} under partially stationary culture. This method was improved by using the mutant Ki-1023 derived from *E. coli* K-12 and by feeding fumaric acid and ammonia during the cultivation of these bacteria.

Enzymatic Production

Enzymatic production of L-aspartic acid from fumaric acid and ammonia by the action of aspartase can be classified as either batch or continuous processes.

Batchwise enzyme reaction

The batchwise reaction is essentially similar to the above-mentioned fermentative method using fumaric acid as a main carbon source, but in this case there is no accompanying growth of microorganisms.

Production of L-aspartic acid from ammonium fumarate using dried cells of *E.coli* No. 2 having high aspartase activity was investigated. In this case, 0.5 g of the dried cells were added to 100 ml of 20% ammonium fumarate solution (pH 7.2-7.4), the mixture was incubated at 37°C for 18h and L-aspartic acid was obtained with a yield of 88.1% from fumaric acid. In this experiment, in which the fermentative broth was used as an enzyme source, L-aspartic acid was released efficiently by the addition of a surfactant such as cetylpyridinium chloride.

The effect of chemical compounds on the preparation of L-aspartic acid was also investigated in detail using the fermentative broth of *Brevibacterium ammoniagenes*. Some surfactants such as cetyltrimethylammonium bromide and cetylpyridinium chloride, higher fatty acids such as oleic acid and linoleic acid, and penicillin were found to enhance L-aspartic acid production.

On the other hand, the fermentative broth of *pseudomonas trefallii* could produce high levels of L-aspartic acid without addition of surfactant, and under optimum conditions 400 g of L-aspartic acid were formed per liter of the reaction mixture.

Production of L-aspartic acid from ammonium fumarate was also investigated using a fumaric acid-assimilating thermophilic bacteria. *Bacillus stearothermophilus* and an α -amino-n-butyric acid-resistant strain of *brevibacterium* sp.

Continuous enzymatic production

Fermentative or enzymatic methods for industrial production of L-aspartic acid can be carried out by batchwise reaction. In order to isolate L-aspartic acid from the reaction mixture, it is necessary

to remove microbial cells and enzyme proteins by pH and/or heat treatment. Even if enzyme activity remains in the reaction mixture, the microbial cells have to be discarded, resulting in uneconomical use of enzyme or microbial cells.

To develop a more efficient continuous production of L-aspartic acid using immobilized aspartase. Among the immobilization methods attempted, relatively active immobilization aspartase was obtained by entrapping it in polyacrylamide gel. The stability of the immobilized enzyme column was investigated by operating it continuously for a long period. As shown in Fig. 20.1, the activity of the column decreased to 50% of the initial value after operation for about 30 days at 37°C. For industrial application of this method, the enzyme has to be extracted from microbial cells. Furthermore, intracellular enzymes are unstable when extracted from the cells, and the activity yield and the stability of the immobilized enzyme are not satisfactory for industrial purposes.

By immobilizing whole cells these disadvantages can be overcome. Among the methods tested, the most active immobilized *E.coli* cells were obtained by entrapping the cells in a polyacrylamide gel lattice.

An interesting phenomenon was observed with the immobilized cells. When freshly prepared immobilized *E. coli* cells were suspended at 37°C for 24-48 h in a substrate solution, their activity increased about 10 times. As this phenomenon is advantageous for continuous production of L-aspartic acid, the activation mechanism was investigated in detail. The apparent enzyme activity was found to be elevated by an increase of membrane permeability for substrate and/or product due to autolysis of the cells in the gel lattice. This phenomenon was similar to the case of the batchwise reaction using the fermentative broth described above, *i.e.* the production of L-aspartic acid was efficiently carried out by the addition of a surfactant to the reaction mixture.

Contaminants such as microbial cells, proteins and so on are not present in the effluent from the column. Therefore, L-aspartic acid of high purity can be obtained in high yield without recrystallization by a very simple procedure such as adjusting the pH of the effluent to the isoelectric point of the acid. As shown in Fig. 20.2, the effluent of appropriate volume is adjusted to pH 2.8

with 60% H_2SO_4 at 90°C and then cooled to 15°C . The L-aspartic acid crystallizes out and is collected by centrifugation or filtration and washed with water. The yield is 90-95% of theoretical.

For industrial application of this technique. We carried out an analysis of continuous enzyme reaction using a column packed with immobilized *E. coli* cells, and the aspartase reactor system was designed. As this aspartase reaction is exothermic, the column reactor used for industrial production of L-aspartic acid was designed as a multi-stage system with cooling.

A comparison of the costs for production of L-aspartic acid by the conventional batch process using intact cells and the continuous process using immobilized cells is shown in Fig. 20.4. The overall production cost by this system was reduced to about 60% of that of the conventional batchwise reaction using intact cells due to the marked increase of productivity of L-aspartic acid per unit of cells, reduction of labor costs due to automation, and an increase in the yield of L-aspartic acid. Furthermore, the procedure employing immobilized cells is advantageous from the standpoint of waste treatment. This was the first industrial application of immobilized microbial cells as a solid catalyst.

To improve this process further, an immobilization technique using x-carrageenan, a polysaccharide isolated from seaweed, was examined. This polysaccharide is composed of β -D-galactose sulfate and 3,6-anhydro- α -D-galactose, which are non-toxic and widely used as food additives.

The aspartase activity and the operational stability of *E. coli* cells immobilized with x-carrageenan were compared with those immobilized with polyacrylamide. The enzyme activity of immobilized cells prepared with x-carrageenan was much higher, and the operational stability was increased by hardening treatment with glutaraldehyde and hexamethylenediamine. Its half-life was 680 days, almost two years. L-Aspartic acid productivities of *E. coli*

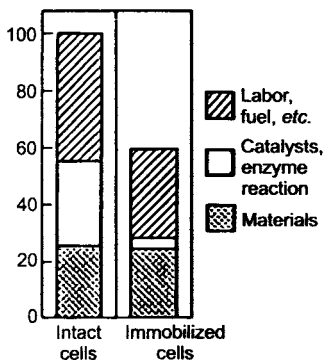


Fig. 20.1 : Comparison of cost for production of L-aspartic acid.

cells immobilized with polyacrylamide and with carrageenan are compared in Table 20.1. The productivity of cells immobilized with carrageenan and hardened with glutaraldehyde and hexamethylenediamine is about 15 times that of the polyacrylamide-immobilised system. With a 1000 liter column, the theoretical yield of L-aspartic acid is 3.4 ton d⁻¹.

Table 20.1 : Comparison of L-Aspartic Acid Productivities of *Escherichia coli* Immobilized with Polyacrylamide and with Carrageenan

Immobilization method ^a	Aspartase activity (units per g cells)	Stability at 37°C (half-life, d)	Relative productivity ^b
Polyacrylamide	18 850	120	100
Carrageenan	56 340	70	174
Carrageenan(GA)	37 460	240	397
Carrageenan (GA+HMDA)	49 400	680	1498

^aGA = glutaraldehyde, HMDA = hexamethylenediamine. ^bProductivity = $S_0 E_0 \exp(-k_d t)$ where E_0 = initial activity, k_d = decay constant, t = operational period.

The continuous production of L-aspartic acid from ammonium fumarate has been investigated in the laboratory scale using *E. coli* cells immobilized with polyurethane. Yokote immobilized aspartase, extracted from *E. coli* cells in the presence of the substrate, to a weakly basic anion exchange resin. Duolite A-7, by ionic binding and investigated the continuous production of L-aspartic acid. The half-life of the immobilized enzyme was found to be 18 days at 37°C and when 2M ammonium fumarate was passed through the column packed with the immobilized enzyme with a residence time of less than 0.75 h and at a temperature of 37°C, the percentage conversion of the substrate solution could be maintained at more than 99% for 3 months.

In addition to the processes described above, other methods of immobilization of aspartase have been carried out. Aspartase extracted from *Escherichia intermedia* was entrapped in cellulose triacetate porous fibers and the enzyme from a thermophilic bacterium, *Bacillus aminogenes*, was immobilized ionically on the above-mentioned Duolite A-7 and then treated with glutaraldehyde.

Utilization of L-Aspartic Acid

L-Aspartic acid and its derivatives are widely used in medicines, foods and cosmetics. Clarification of the specific physiological role of L-aspartic acid has brought about its therapeutic application. The importance of K^+ and Mg^{2+} in myocardial cells function has been the subject of increased interest, and L-aspartic acid was found to be an ideal carrier substance for these ions. Thus potassium magnesium aspartate is widely used to assist recovery from fatigue, heart failure, and liver disease of diabetes. In addition, potassium aspartate is used for alleviating leg cramp syndrome and liver disease, and ferrous aspartate is used for treating anemia.

Some L-aspartic acid salts are used as surface active agents and for the treatment of keratodermatitis. The addition of L-aspartic acid or its derivatives, together with vitamin B_6 , to cosmetic bases has been suggested to be effective in preventing ageing of the skin or revitalizing old skin.

In food processing, although L-aspartic acid is acidic, its neutralized solution is tasty. Sodium aspartate is used as a seasoning in orange juice. It is known that there is a synergistic action in terms of taste between amino acid and mononucleotides. The taste of sodium inosinate, which was identified as a taste substance of Katsuobushi (dried bonito), is not strong in itself, but is enhanced by the addition of monosodium L-aspartate.

In the course of studies on the synthesis of gastrin, a hormone-stimulating gastric juice, Mazur *et al* (1969, 1970) found that an intermediate of gastrin synthesis, L-aspartyl-L-phenylalanine methyl ester, showed very strong sweetness. The dipeptide Aspartame was found to be about 200 times sweeter than 4% sucrose in an aqueous solution and to be low in calories. Aspartame is now being developed by several companies and will become one of the important materials in the market for artificial sweeteners.

21

Threonine

Threonine, or α -amino- β -hydroxy-n-butyric acid, was first isolated from an acid hydrolyzate of oat glutelin but no nutritional significance was attributed to the compound at the time, nor was there any concept of its optical configuration.

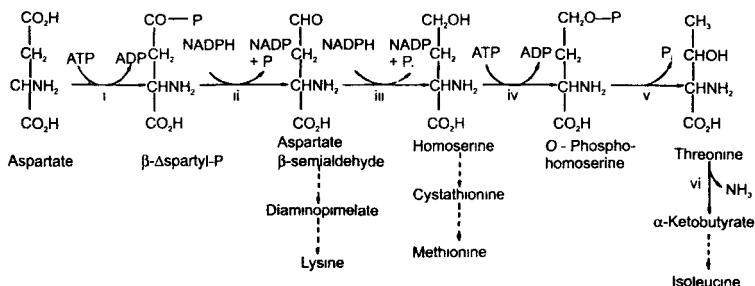


Fig. 21.1 : Biosynthetic pathway of the aspartate family of amino acids; i, aspartate kinase; ii, aspartate-semialdehyde dehydrogenase; iii, homoserine dehydrogenase; iv, homoserine kinase; v, threonine synthase; and vi, threonine dehydratase (deaminase)

As threonine contains two asymmetric centers, there are four stereoisomers of the molecule. Of these isomers, only L-threonine is effective in nutrition. A deficiency of L-threonine produces stunted growth followed by metabolic disorders throughout the body, such as decrease in bodyweight and anaemia, and may induce functional disorders of the liver.

Regulatory Mechanisms of Threonine Biosynthesis in Several Microorganisms

As mentioned above, it is necessary to circumvent the regulatory mechanisms operating in the threonine biosynthetic pathway in order to accumulate a large amount of threonine in the

fermentation broth. The mode of regulation in the threonine synthetic pathway seems to vary in different microorganisms. The regulatory mechanisms of threonine biosynthesis in two typical threonine producing bacteria strains of *E. coli* K-12 and *Brevibacterium flavum*, are shown in Fig. 21.2.

In *E. coli* K-12, three aspartate kinases are known, one competitively inhibited and repressed by L-threonine, the second one not inhibited by L-methionine but repressed by it, and the third one non-competitively inhibited and repressed by L-lysine. In contrast to this great complexity of the aspartate kinase system in *E. coli* in *Brevibacterium flavum* only one aspartate kinase is known, and its activity is regulated by a feedback inhibition with L-threonine and L-lysine, but not by end-product repression. In addition, no evidence showing the operation of repressions by L-threonine, L-isoleucine or L-lysine in the synthetic pathway of the aspartate family of amino acids has been obtained so far. This fact suggests that regulatory mechanisms in threonine biosynthesis may vary in different microorganisms. Therefore, it is a prerequisite to elucidate the regulatory mechanism of threonine biosynthesis in the individual microorganism which is selected as the threonine producer.

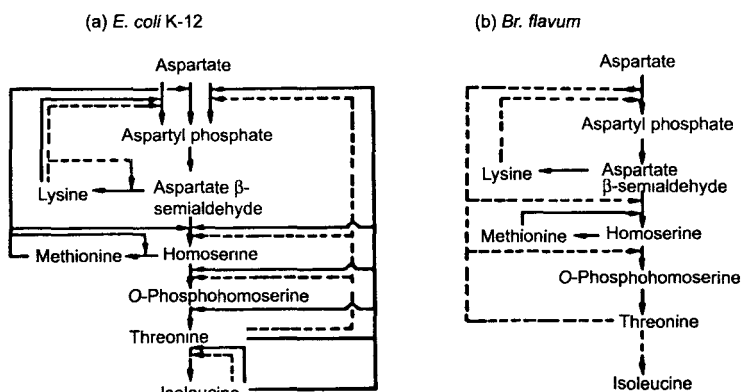


Fig. 21.2 : Summary of feedback regulation in the biosynthesis of the aspartate family amino acids in (a) *E.coli* K-12 and (b) *Brevibacterium flavum*: feedback inhibition \rightarrow , repression \rightarrow

Selection of Threonine-Producing Bacteria

Escherichia coli

It was first reported that an *E. coli* mutant resistant to α -amino- β -hydroxyvaleric acid (AHV), an analogue of threonine, excreted L-threonine into media, and that homoserine dehydrogenase activity of the mutant was less sensitive to feedback inhibition of threonine than that of the parental strain. Based on this observation, an attempt to isolate threonine-producing *E. coli* mutant resistant to AHV was made. AHV resistant mutants, both spontaneous and induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) treatment of *E. coli* PB-8, were obtained and the resistant colonies were isolated on a medium containing 1 mg ml⁻¹ of AHV after 48 h incubation at 37°C. Among many AHV resistant mutants obtained, one of the isoleucine auxotrophs, strain β I-67, produced about 4.7 mg ml⁻¹ of threonine, and one of the methionine auxotrophs, strain β IM-4, derived from strain β I-67 produced about 6.1 mg ml⁻¹ of threonine when cultured in a medium containing 3% glucose supplemented with 100 μ g ml⁻¹ of L-isoleucine and L-methionine.

The threonine production by auxotrophs derived from AHV sensitive and resistant mutant strains is shown in Table 21.1. It is of interest that simple methionine auxotrophs accumulated threonine, but simple isoleucine or lysine auxotrophs did not. These results suggest that the alteration of the sensitivity of homoserine dehydrogenase to the threonine inhibition as is the case with AHV resistant mutants, or formation of another threonine insensitive homoserine dehydrogenase and aspartokinase as occurs with methionine auxotrophs, would be essential for threonine production in *E. coli*. As seen in the cases of strains PBMI-13 and PBMI-14, the addition of isoleucine requirement to methionine auxotrophs enhanced their threonine production, although simple isoleucine auxotrophs did not accumulate threonine. This result suggests that isoleucine repressible enzymes in the pathway, other than homoserine dehydrogenase, are rate limiting for threonine production in these methionine auxotrophs.

Some treated various bacteria with UV light or nitrosoguanidine (NG) to derive auxotrophic mutants, which were screened for their ability to produce threonine. Among the auxotrophs obtained, triple auxotrophs[DAP⁻(a-e=diaminopimelic acid), Met⁻, Ile⁻] of *E. coli* and their isoleucine revertants were screened for

their threonine productivity. One of the isoleucine revertants, KY 8280, was further studied to determine optimal culture conditions to attain a maximal accumulation of threonine.

Among various carbon sources tested, fructose was found to give the best production of threonine. Threonine accumulation during the fermentation in a 5 l jar fermenter with a medium containing 7.5% fructose, DL-methionine ($50 \mu\text{g ml}^{-1}$) DAP ($100 \mu\text{g ml}^{-1}$) and isoleucine ($25 \mu\text{g ml}^{-1}$) was examined. Cell concentration reached a maximum in about 48 h, after which the concentration of L-threonine increased rapidly, with a concurrent consumption of fructose and attained a level of 13.8 mg ml^{-1} in 120 h. Some described threonine production by DAP and DAP plus methionine auxotrophs of *E. coli*.

Table 21.1 : Threonine Accumulation by Auxotrophs derived from AHV sensitive and-resistant *E.coli* Strains

Strain	Nutritional requirement	AHV resistance ^b	Growth (O.D. ₅₆₂)	Final pH	L-Thr (g/l)
PB-8	—	—	0.362	4.8	0.01
PBI-6	Ile	—	0.251	4.8	0.01
PBI-1	Ile	—	0.241	5.0	0.01
PBL-1	Lys	—	0.134	5.2	0.09
PBM-1	Met	—	0.369	4.9	1.74
PBM-28	Met	—	0.432	4.8	1.34
PBM-13	Met,Ile	—	0.348	4.8	2.99
PBML-14	Met,Ile	—	0.310	4.8	2.67
β-101	—	+	0.424	4.8	1.47
β-133	Met, Ile	+	0.393	4.8	1.20
βL-67	Ile	+	0.392	4.8	4.69
βM-7	Met	+	0.375	5.2	3.78
βM-9	MeT	+	0.430	5.0	3.59
βL-1	Lys	+	0.221	5.2	0.11
βIM-4	IIE, Met	+	0.350	5.0	6.10
βIML-6	IIE, Met, Lys	+	0.215	5.2	3.40

^b — and + represent AHV sensitive and resistant respectively. Fermentation medium was supplemented with 100 mg l^{-1} of required L-amino acids.

Scientists selected threonine producing auxotrophs of *E. coli* C-6 by treating them with UV radiation or 'NG. One of the

methionine-valine auxotrophs, strain No. 234, was found to produce a large amount of L-threonine in a culture medium supplemented with DL-methionine ($60 \mu\text{g ml}^{-1}$) and L-valine ($400 \mu\text{g ml}^{-1}$). The yield of threonine was greatly dependent on the temperature of incubation. Higher yields of threonine were obtained when the temperature was kept at a relatively high level ($38\text{--}40^\circ\text{C}$). In addition, it appears that there is a close relationship between threonine production and the cell yield. When the yield of cells was repressed, the accumulation of threonine increased with increasing temperature. The yield of L-threonine in the broth of the glucose medium was also enhanced by the temperature shift-down technique, in which the culture temperature was shifted from 38 to 28°C at 17 h after the start of cultivation. This procedure was particularly effective in maintaining a high level of threonine production throughout the fermentation.

The addition of an antibiotic, borrelidin, together with L-aspartic acid to the culture medium of *E.coli* strain No.234 increased threonine accumulation to about 15 mg ml^{-1} . There was more than five-fold derepression of the threonine biosynthetic enzymes when *E.coli* was grown in the presence of trace amounts of borrelidin, therefore the stimulative effect of borrelidin on the threonine accumulation may possibly be caused by an increase in the activities of the enzymes responsible for threonine biosynthesis.

Serratia marcescens

Scientists attempted to isolate threonine producers from the mutant strains of *Serratia marcescens* which had been used by them for isoleucine production studies. These mutants were known to lack both feedback inhibition of threonine deaminase and repression of isoleucine biosynthetic enzymes, and, therefore, produce a large amount of isoleucine in a medium containing glucose and urea. These facts suggest that a mutant strain of *S. marcescens* might be attractive for threonine production, if the absence of threonine degrading enzymes could be included in the mutant strains. Thus, AHV-resistant mutants were selected from cells of the isoleucine auxotroph of *S. marcescens* strains D-60 by treating it with NG.

The selection was carried out by spreading the mutated cells of strain D-60 on a minimal agar plate containing glycerol as a carbon source and high concentration of AHV, L-isoleucine, L-methionine

and L-lysine. These three natural amino acids were added to select mutants lacking feedback controls to a greater extent. Among the AHV-resistant mutants obtained, three representative mutants were tested for threonine production. As shown in Table 21.2, the parent strain D-60 produced minimal levels of threonine in a medium containing a limiting amount or an excess of isoleucine; Strain HNr21 produced approximately 11 mg ml⁻¹ of threonine on addition of a limiting amount of isoleucine. This production was decreased with a higher concentration of isoleucine, owing to its repressive effect on threonine synthetic enzymes. Strain HNr59 also produced threonine to a lesser extent than that of strain HNr21, owing to the feedback inhibition of aspartate kinase. Strain HNr53 produced a trace amount of threonine, a result of feedback inhibition of aspartate kinase and homoserine dehydrogenase. Strain E-84, a DAP auxotroph derived from strain HNr59, produced an increased amount of threonine (13 mg ml⁻¹) with the addition of appropriate amounts of DAP and lysine. In this auxotroph the lysine-sensitive aspartate kinase might be physiologically released from feedback controls by limiting the lysine source. This indicates that a genotype lacking feedback control of the lysine-sensitive aspartate kinase would increase the threonine productivity of *S. marcescens* strains.

Table 21.2 : Theonine Production by AHV-resistant Mutants of *S. marcesceus*

Strain	Addition of L-isoleucine to the medium (mg ⁻¹)	Growth (g dry wt l ⁻¹) at :			L-Thronenine producced (gl ⁻¹) at:				
		48 h	72 h	96 h	120 h	48 h	72 h	96 h	120 h
D-60	2	5.8	7.4	12.8	23.8	0.1	0.1	0.3	0.3
	10	17.2	27.3	25.2	19.4	0.1	0.1	0.1	0.1
HNr21	2	7.0	11.2	19.0	18.1	1.9	7.4	8.9	10.9
	10	16.8	22.7	24.2	20.0	0.9	3.6	4.2	3.7
HNr53	2	1.6	7.3	11.3	18.3	0.1	0.3	0.3	0.3
	10	12.8	18.3	19.4	18.3	0.3	0.3	0.3	0.3
HNr59	2	3.2	13.3	26.1	28.9	1.2	2.9	4.8	5.0
	10	8.4	25.3	27.4	26.0	1.0	3.0	4.6	5.1

Brevibacterium

It was observed that threonine and homoserine auxotrophs derived from *Brevibacterium flavum* No. 2247, a glutamate producing

bacterium, accumulated L-lysine in the broth to a level high enough to be used for industrial scale production. Since homoserine dehydrogenase of this strain was inhibited strongly by L-threonine as in the case of *E. coli*, it was expected that AHV-resistant mutants of glutamate producing bacteria would produce as much threonine as lysine.

Some selected threonine producers from AHV-resistant mutants of *Br. flavum* No. 2247. The best producer, strain B-183, was isolated from a plate containing 5 mg ml⁻¹ of AHV and accumulated 10.5 mg ml⁻¹ of L-threonine. The culture medium used in the threonine production contained per liter 100 g glucose, 30 g ammonium sulfate, 15 g KH₂PO₄, 0.4 g MgSO₄ · 7H₂O, 2 p.p.m. Fe²⁺, 2 p.p.m. Mg²⁺, 200 µg D-biotin, 300 µg thiamine HCl, 4 ml Mieki (a mixture of amino acids) and 50 g CaCO₃ (pH 7.2).

In these experiments, it appeared that mutants resistant to higher concentrations of AHV accumulated larger amounts of threonine than those less resistant to AHV. Thus, further selection of the AHV mutants derived from strain B-183 by treating it with NG was carried out. Among the mutants tested, about twenty strains produced more threonine than did the parent strain. Out of these mutants, strains BB-82, BB-69 and BB-24 accumulated 13.5, 12.9 and 11.9 mg ml⁻¹ of threonine with the culture medium described above.

The time courses of growth, pH and threonine production using BB-82 strain are given in Figure 21.3. Maximal levels of threonine accumulation (13.5 mg ml⁻¹) were obtained between 60 and 70 h, just after reaching a plateau of the growth curve. In the broth of the strains resistant to AHV, in addition to L-threonine, a significant amount of L-homoserine (2.7 mg ml⁻¹) and lower amounts of other amino acids such as L-valine, L-isoleucine, L-leucine, DL-alanine and glycine also accumulated. It is, of course, desirable to reduce the accumulation of any other amino acids for industrial production of threonine.

To increase threonine production by *Br. flavum*, further attempted to get methionine and lysine auxotrophs from strains BB-69 and BB-2, threonine producers derived from *Br. flavum* No. 2247 mentioned above. Five lysine auxotrophs and 76 methionine auxotrophs were isolated. None of the lysine auxotrophs produced more threonine than the parental strain; one methionine auxotroph,

strain BBM-21, accumulated more threonine than the parent strain when cultured in a medium containing 200 mg ml^{-1} of L-methionine. The maximum production of threonine (17.5 mg ml^{-1}), which was 50% more than with the parental strain, was obtained when the initial concentration of methionine was $750\text{--}1000 \text{ } \mu\text{g ml}^{-1}$; further addition of methionine decreased the level of threonine. The maximum cell concentration was attained at more than $250 \text{ } \mu\text{g ml}^{-1}$ of methionine. It is noteworthy that the addition of methionine markedly reduced homoserine accumulation.

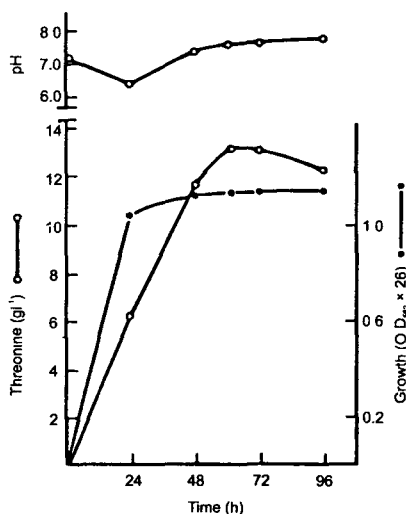


Fig. 21.3 : Time course of threonine production in *Br. flavum* (Shiio and Nakamori, 1970)

It has been shown that the homoserine dehydrogenase of threonine producers selected as AHV resistant mutants, and the aspartate kinase of lysine producers selected as AEC resistant mutants, derived from *Br. flavum* are genetically desensitized to feedback inhibition by their end products, L-threonine and L-threonine plus L-lysine, respectively. As these two enzymes are involved in the synthesis of threonine, it seems useful for threonine overproduction to derive mutants in which both aspartate kinase and homoserine dehydrogenase are desensitized to feedback inhibition by L-threonine plus L-lysine and L-threonine, respectively.

Scientists have selected AHV-resistant mutants from the AEC resistant strains of *Br. flavum* FA-1-30 and FA-3-115, which has been isolated as lysine producers. Many resistant mutants isolated from a plate containing 2 mg ml⁻¹ of AHV accumulated lysine as well as threonine, while none of the mutants from plates containing 3 or 5 mg ml⁻¹ of AHV accumulated lysine. The best producer, FAB-44, isolated from NG-treated FA-1-30 accumulated 15 mg ml⁻¹ of threonine in a medium containing 5 mg ml⁻¹ of AHV.

Other Microorganisms

In addition to the threonine producing bacteria mentioned above, auxotrophic mutants of various bacteria have been examined for their ability to accumulate threonine. Fifteen strains of bacteria representing seven families were mutagenized to derive auxotrophs of amino acids directly related to threonine biosynthesis, i.e. methionine, isoleucine, lysine and α , ϵ -diaminopimelic acid. Among the isolated auxotrophs, those which produced appreciable amounts of L-threonine were found in *Aerobacter aerogenes*, *S. marcescens* and *E. coli*, all of which belong to the family Enterobacteriaceae.

Auxotrophic mutants of *Arthrobacter paraffineus* were also selected as threonine producers. Among them, five mutants produced above 12 mg ml⁻¹ of threonine with the medium containing 10% *n*-paraffins (C₁₂-C₁₄ rich) and 2 mg ml⁻¹ of DL-methionine.

Threonine Producers Obtained by Genetic Manipulation

Microbial strains having a high productivity of a specified amino acid require multiple mutation leading to (i) release of feedback controls of the amino acid biosynthetic pathway, (ii) defects in enzymes degrading the amino acid, (iii) defects in enzymes involved in the flow of biosynthetic precursors to side pathways, (iv) enhancement of the formation of the precursors, (v) prevention of the accumulation of by-products, and (vi) genetic stabilisation of the amino acid producing strain.

Experimental procedures to obtain better strains for amino acid production by using genetic techniques such as transduction, transformation and conjugation have been developed. Scientist have applied a transductional technique for construction of threonine producing strains of *s. marcescens*. This bacterium has the

advantages that the regulatory mechanisms operating in the biosynthetic pathway of threonine is very similar to that of *E. coli* and that bacteriophage BP-20 is known to be an effective transducing phage in *S. marcescens*. In the threonine synthesis pathway, aspartate kinases and two homoserine dehydrogenases. All these enzymes are feedback-controlled by threonine, isoleucine, methionine and lysine, singly or in combination (Fig. 21.4). With phage PS-20-mediated transduction, some transferred the *thrA1* and *thrA2* mutations (*thrA1*: lack of feedback inhibition of threonine-sensitive aspartate kinase; *thrA2* and *thrA22*: lack of feedback inhibition of homoserine dehydrogenase I and II, respectively) into strain E-60 lacking repression by the threonine operon (*hnr-1*, lack of repression of threonine) in the initial stages of the study. One of the transductants, strain T-570, produced about 8 mg ml⁻¹ of L-threonine in a medium containing sucrose and urea. This strain was assumed to be a recombinant carrying *thrA1*, *thrA2* and *hnr-1*.

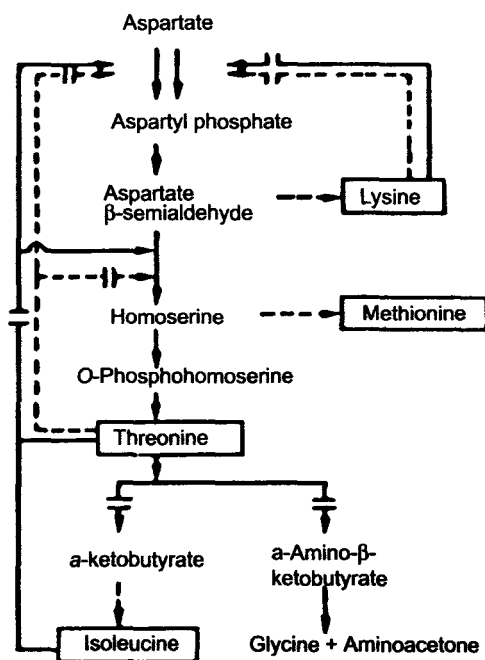


Fig. 21.4 : Regulation of biosynthesis and degradation of threonine in *S. marcescens*: feedback inhibition: → repression: →

Subsequently, an additional regulatory mutation lacking both feedback inhibition and repression of lysine-sensitive aspartate kinase was introduced into strain T-570 using strain AECr174 as donor.

A further improvement in the transductional construction of threonine-producing strains of *S. marcescens* has been achieved. The six regulatory mutations were independently selected by isolating mutants resistant to analogs of threonine, methionine and lysine. The genealogy of the main strains used in the transductional experiments is given in Figure 21.5. Strain Mu-910 was isolated from strain 8000 (wild-type) and lacks threonine dehydrogenase. Strain D-60 was derived from strain Mu-910 as an isoleucine auxotroph, lacking both threonine dehydrogenase and threonine deaminase. The strains carrying *thrA₁I* and *thrA₂I* mutations lack feedback inhibition of aspartate kinase I and homoserine dehydrogenase I. Mutations *hnrA1* and *hnrB2* lead to depression of these two enzymes. Mutation *hnrA1* is linked to the *thr* locus, but

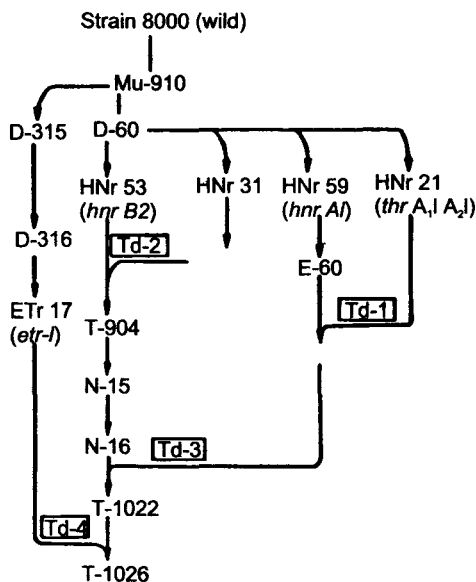


Fig. 21.5 : Genealogy of main strains used in the transductional construction of threonine-producers of *S. marcescens*: Td, transduction.

mutation *hnr B2* is not. A mutation causing lack of both feedback inhibition and repression of aspartate kinase III is denoted as *lysC₁*. Mutation *etr-I* causes derepression of aspartate kinase II and homoserine dehydrogenase II.

By four transductional crosses as indicated in Figure 21.5 (Td-1-4), the mutations described above were combined in a single strains D-60 defective in threonine-degrading enzymes. Of the transductants selected, strain T-1026 was found to be relieved of both feedback inhibition and repression of three aspartate kinases and two homoserine dehydrogenases owing to the six regulatory mutations. This strain produced about 40 mg ml⁻¹ of threonine, whereas the other strains, lacking more than one of the six mutations, produced less than 30 mg ml⁻¹ of threonine (Table 21.3). Strain T-1026 was further improved to strain P-200, which had both isoleucine and methionine bradytrophies. Strain P-200 accumulated more than 40 mg ml⁻¹ of threonine in a simple fermentation medium containing sucrose and urea. This strain was quite stable and the cultured medium contained no detectable amounts of amino acids other than threonine.

Table 21.3 : Threonine Production by Transductionally Constructed Strains of *S. marcescens*^a

Strain	Genotype						L-Thr produced (gl-1) ^b
	Lys C	A ₁	thr A ₂	A	hnr B	etr	
D-60	+	+	+	+	+	+	<0.1
HNr21	+	/	/	+	+	+	4.2
HNr53	+	+	+	+	2	+	0.7
HNr59	+	+	2	/	+	+	4.3
AECr174	/	+	+	+	+	+	7.4
Etr17	+	+	-	+	+	/	7.5
T-570	+	/	/	/	+	+	8.8
T-904	/	+	+		2	+	10.2
T-1021	/	+	+	+	2	/	28.9
T-1025	/	/	/	/	2	+	25.8
T-1026	/	/	/	/	2	/	40.3

^b Medium: 15% sucrose, 1.5% urea, 0.05% (NH₄)₂SO₄, 0.1% MgSO₄, 7H₂O, 0.2% C.S.L., 10mM Ile, 10mM met and 1% CaCO₃, 96-120 h.

Discussion

Among the enzymes concerned with the synthesis of threonine in bacteria, aspartate kinase and homoserine dehydrogenase are considered to play a dominant role in feedback controls of threonine synthesis. Recently, it was demonstrated that aspartate kinase I activity in *E. coli* is associated with a second activity, homoserine dehydrogenase I, carried on a Bifunctional enzyme, which is a tetramer containing a single kind of subunit. The polypeptide chain contains two distinct functional regions; one carried by the amino-terminal portion yields a threonine-sensitive aspartate kinase devoid of homoserine dehydrogenase activity and the other carried by the carboxy-terminal portion exhibits only homoserine dehydrogenase activity. The bifunctional enzyme, aspartate kinase I-homoserine dehydrogenase I, is specified by the *thrA* gene in *E. coli* and multivalently repressed by threonine plus isoleucine. When selecting threonine producers, therefore, it is absolutely necessary to obtain mutants in which aspartate kinase I-homoserine dehydrogenase I has become insensitive to threonine. The feedback controls by isoleucine could be avoided by introducing isoleucine auxotrophy into the mutant. As shown in Table 21.1 one of the *E. coli* AHV resistant mutants, strain β -101, accumulated about 1.9 mg ml⁻¹ of L-threonine, and an isoleucine auxotroph β I-67, derived from β -101 produced about 4.7 mg ml⁻¹.

In *E. coli* K-12, aspartate kinase II is also associated with a homoserine dehydrogenase activity in a bifunctional protein. Neither activity is inhibited by methionine or any other amino acid of the aspartate family. The enzyme is, however, repressed by methionine. Aspartate kinase II-homoserine dehydrogenase II is a dimer of identical subunits. Thus, we can anticipate further enhancement of threonine production by using a methionine auxotroph derived from the β I-67 strain described above. In fact, an isoleucine and methionine double auxotroph, strain β IM-4, was selected and shown to accumulate threonine more than the parent strain β I-67.

Aspartate kinase III in *E. coli* appears not to be associated with any additional activities. It is a dimer of identical polypeptide chains and is both repressed and inhibited by lysine. The addition of lysine requirement to isoleucine plus methionine auxotrophs, however, was rather inhibitory for threonine production (see strain

β IML-6 in Table 21.1). A possible explanation of this negative effect could be that the lysine limiting conditions may relieve the enzyme of the first step in the lysine biosynthetic pathway from feedback inhibition, causing the consumption of much larger amounts of the common intermediate to the lysine pathway than occurs in the parent strains, and may thus decrease threonine production. To avoid the negative effect of lysine limitation on threonine production, it is necessary to obtain a lysine auxotroph lacking the first step enzyme in the lysine pathway.

Most attempts to obtain better threonine-producing strains have been performed by adding sequentially required mutations to a single strain. However, this method does not always to obtain mutants that completely lack individual regulatory mechanisms because of cross-resistance to analogs or the reversion of prior mutations. These problems have been solved by applying a transduction technique. Using *S. marcescens* individual mutations to relieve feedback controls by threonine, isoleucine, methionine and lysine can be independently selected, and combined by contrasducing with appropriate selecting markers. Thus, the constructed strain of *S. marcescens* T-1026 exhibited the highest productivity of threonine (Table 21.3).

Cocoa Fermentation

Cocoa fermentation is necessary for the formation of flavor precursors, however, true chocolate flavor is developed only in roasting. In addition to its role in flavor production, cocoa fermentation prevents cocoa butter degradation by killing the bean before changes associated with germination begin and the fruit pulp is degraded, which makes bean drying easier.

Cocoa fermentation is a mixture of external microbiological processes, chiefly characterized by the production of ethanol and acetic acid from carbohydrates and an internal autolytic process involving cocoa bean enzymes. The term "cocoa fermentation" embraces all of the dictionary definitions of fermentation, a microbial process, enzyme action, and flavor improvement.

Agronomic and Botanic Background

Theobroma cacao Taxonomy

The cocoa of commerce is the dried and more or less fermented seeds of *Theobroma cacao* L. In the fermentation literature, cocoas are divided into a number of groups based on pod shapes, seed characteristics and taxonomic considerations. Scientists divided all cocoas into three groups based on Venezuelan trade names. These are now familiar to all of the cocoa industry as criollo for astero and trinitario. Criollos are characterized by ten deep furrows, thin pod walls, large, nearly round seeds with white or pale violet cotyledons. There is very little criollo cocoa in world trade.

Forastero cocoa has ten shallow furrows on the pods, pod walls are thick and may be woody, the seeds are flatter and, with minor exceptions, of a deep purple hue. The majority of the world cocoa supply is forastero.

Trinitario cocoa consists of hybrids between various criollos and forasteros. Trinitarios, because of recrossing and random segregation of traits in higher order filials (Fn) and unless planted as selected clones, constitute hybrid swarms exhibiting a wide range of combinations of criollo and forastero characteristics. The amount of trinitario cocoa in trade is intermediate between the other types.

Botanical Description of the fruit

The fruit of the cocoa tree is a berry, but is commonly referred to as a pod in English, a mazorca in Spanish, or a cabosse in French. A berry is defined as - superior or inferior, indehiscent, many seed fruit, usually with a fleshy pericarp. The pericarp consists of three layers - an epicarp, a more or less lignified mesocarp, and an endocarp. Only the endocarp is important in cocoa processing, as this is the pulp. The seeds are arranged around the central placenta. These are in five rows derived from the five locules of the ovary. The fruit takes approximately 140-205 days post-pollination to reach maturity and ripeness.

Fruit Growth and Maturation

At harvest time, the pods change color from green or red to yellow or orange. At this time, the endocarp or pulp surrounding the seeds is in a greater or lesser state of senescence. In overripe pods, the amount of pulp is small and it appears to be dry. In very overripe pods it is beginning to brown as well. Under ripe pods have a dense, white endocarp of parenchyma cells.

Biochemical changes accompany the physical changes in the endocarp. We found a qualitative difference in pulp sugars of pods from 25 to 170 days old, as well as an increase in sugar concentration as the pods ripened.

The pods were then dissected into three parts; the pericarp and mesocarp, the husk endocarp, and the seeds with pulp. A random sample of about 10 g of seeds was taken for sugar analysis. Fructose, glucose, and sucrose were determined by thin layer chromatography. The husk fractions were used for pectin analysis.

Pulp sugar content undergoes both qualitative and quantitative change as the pods mature.

The approximate amount of sugars found in fresh pulp of pods of various ages is given in Table 22.1.

Table 22.1 : Pulp Sugar from *Theobroma cacao* Fruit of Varying Ages as% of fresh weight

Age of Fruit	Glucose (%)		Fructose(%)		Sucrose (%)	
	A ^{a)}	B ^{b)}	A	B	A	B
25 Days	1.17		0.71		—	
46 Days	0.82		0.50		—	
57 Days	0.97		0.89		—	
73 Days	0.82		0.78		0.05	
87 Days	0.67		1.21		0.51	
3.5 Months		—		—		5.0
107 Days	0.55		0.82		0.88	
4.0 Months		—		—		6.3
4.5 Months		1.3	—	—		6.5
143 Days	0.64		0.66		1.56	
5.0 Months		3.3		trace		3.5
5.5 Months		5.5		2.2		2.0
170 Days	1.09		1.06		2.90	
6.0 Months		6.0		2.70		1.70
6.5 Months		9.3		—		1.25

Water content of cocoa pulp can vary markedly. This is reflected in the percentage conversion of wet unfermented to dry fermented cocoa.

The degree of pod ripeness has been observed to have a significant effect on yield with the best yields obtained from ripe pods, slightly less favorable yields from overripe pods, and very poor yields from unripe pods. Fermenting overripe fruit may lead to cocoa with very brittle shells.

Description of Embryo Anatomy

The seed at maturity consists of the testa, a very thin remnant of the endosperm and the embryo. We found protease, catalase, and polyphenol oxidase in the endosperm. Apparently, the peroxidase and oxidase are found in different cell types in the bundle sheaths. The endosperm also contains large amounts of thiamine, an inhibitor of polyphenol oxidase. The embryo constitutes about 88% of the seed weight. The testa is about 10-14% of the seed weight and contains an invertase. The embryo is composed of two cotyledons

and an embryonic axis. The axis is less than 1% of the embryo weight and consists chiefly of hypocotyl. The cotyledons are composed of an epidermis, ground parenchyma, and vascular tissue. The ground parenchyma is the major tissue of the cotyledon. The thin walled cells are compactly arranged and intercellular space is lacking. Three types of parenchyma cells can be identified. They are food storage cells, polyphenol storage cells, and mucilage cells. The food storage cells are most numerous. These cells are densely packed with oil droplets, starch and aleurone grains. The polyphenol storage cells are highly vacuolate. These vacuoles can be isolated from dried beans and they contain all of the polyphenols including anthocyanins except for a portion of the epicatechin. They also contain purine alkaloids. These cells make up about 10% of the cotyledons. These cells are found scattered in the parenchyma and are found in more orderly fashion beneath the epidermis in parallel rows or in rows perpendicular to the epidermis.

Environmental and Genetic Effects

Very little is known with certainty with respect to environmental and genetic effects on seed chemistry and the significance of any differences on fermentation. Some states that soils and climate have no effect on cocoa seed flavor characteristics.

Environment is known to have an effect on cocoa butter fatty acid saturation. Seeds maturing in cooler seasons have a higher content of unsaturated fatty acids. Other temperature effects have not been noted, but cannot be ruled out, such as pulp sugar concentration, titratable acidity, and pH of pulp.

Cocoas of different origins are known to differ in fat, protein, alkaloid, and starch contents. These data are usually taken from samples of commercial lots. The lack of controls on these samples does not allow separation of genetic, environmental, or processing effects. The most striking differences in cocoa composition are the color differences. Anthocyanins make up about 0.5% of the dry weight of forastero seeds but are not found in the criollos.

Anthocyanins are inherited dominant to the colorless situation. The location of the defect in the synthetic pathway is not known but criollo cocoas produce the chemically related leucoanthocyanidins.

Harvesting

Cocoa pods are indehiscent and do not absciss. Therefore, they must be removed from the trees and the seeds must be removed from the pods manually. The fruit peduncle must be cut with a sharp instrument in order to avoid damage to the tree. Pods are often gathered up and broken in one or more spots on the farm and beans are transported to the fermentation site.

Alternatively, the pods are collected and stored for up to a week before opening them. The storage of pods may also allow the less ripe pods to ripen further and promote a speedier, more uniform fermentation. When pods are broken directly into fermenting boxes or other fermentation apparatus instead of being broken in small lots in the field and transported to the fermentation site, the earliest phases of the fermentation may differ. The yield percentage was also highest from overripe pods. Both ripe and overripe pods gave a greater yield of fermented beans than underripe pods.

Germination

A period of incipient germination is important to the development of flavor precursors during fermentation. Germination is the emergence of the radical from the seed. This technical definition of germination is often confused by those unfamiliar with plant science with seedling emergence. Thus, with ripe seeds, germination may require 1 or 2 days and seedling emergence, 1-2 weeks, under good conditions. The very early stages of germination such as the decrease in concentration of inhibitors, gene repressors, increased concentration of some enzymes may occur, but whether or not this is important to the development of flavor precursors, is not known.

Anatomical studies of germination revealed that the changes of the first 24 hours occurred primarily in the hypocotyl. During this period, the epicotyl and root meristem seemed to be dormant. Starch grains disappeared from the hypocotyl cortex and pith and vascularization continued. Pith cells begin to senesce, cortical cell walls thicken, and mucilage cells begin to mature. The axis elongates from about 7 mm to 10 mm passing through the micropyle. During the second 24 hours, the root meristem becomes active. What effect these changes might have on the quality of cocoa is not known. Because these observations were made on seeds

removed from their testas and sown in course sand, it is difficult to compare this material with fermenting beans, however, it is likely that the peeled beans develop more rapidly than beans in fermenting masses.

Chemical studies of germination (ROESCH et al., 1961) show that large decreases in fat, starch, and protein occur in the cotyledons over a period of several days.

Polyphenol storage cells remain intact during germination and subsequent development of the cotyledons. Over the period of time that corresponds to fermentation time, little, if any, anthocyanin, permanganate oxidizable substance, or alkaloid is lost from the cotyledons

If germination does occur, it reduces the value of the cocoa by reducing the fat content and opening the testa. A small disc of shell covering the micropyle is loosened by enzymatic action and pushed free by the lengthening embryonic axis.

Fermentation Methods

Four general methods in use. These were: a) curing on the drying platform; b) fermentation in heaps on the ground; c) fermentation in baskets; and d) fermentation in boxes. Tray fermentation is another technique occasionally used to prepare cocoa for market.

Curing on Drying Platforms

This technique is confined almost entirely to Ecuador for the production of Arriba cocoa. The pods are broken and the fresh bean are heaped onto the drying platform. The beans are customarily spread out during the day and heaped during the night. During the palm main crop, a fine product is obtained, however, the quality is reported to be much lower during the minor crop.

Basket Fermentation

This method is used primarily in West Africa. The wet beans are placed in woven baskets and covered with palm leaves. The sweatings can drain through the sides and bottom of the baskets. Using batches of 20 to 200 kg, a satisfactory temperature is produced by the third day of fermentation. The fermentation is continued six to seven days and the cocoa is mixed daily or every

other day by transferring the beans from one basket to another. This method reportedly produces satisfactory cocoa and is particularly suited to the small producer.

Heap Fermentation

This simple method of fermentation is, perhaps, the most commonly used method on smaller farms. The wet beans are heaped on a layer of banana leaves and covered with the same material. It was found advisable to place a circular pattern of sticks under the banana leaves to raise the heap slightly off the ground. To ensure even fermentation and discourage surface mold growth, the heap should be mixed every other day.

Box Fermentation

This method is generally used on larger farms or plantations as it is suitable for large quantities of cocoa. The boxes are constructed of local hardwoods and vary widely in dimensions. A typical size is 1 m × 1 m × 1 m. The boxes must have holes in the floor or be constructed with slatted floors providing spaces through which the sweatings can drain. The box must be filled in one day to prevent uneven fermentation. Often partitions can be added to a larger box to reduce its size to ferment smaller batches. The duration of fermentation varies from four to seven days. The beans are turned over from one box to another. The exact sequence of turnings varies from location to location. With proper attention to detail, fine cocoa can be produced with this method.

Tray Fermentation

We developed a system for fermentation of cocoa in a stack of trays based on the observation that beans at the surface of a heap turned brown faster than those at the center' of the heap. The fresh seeds (45.5 kg/tray) are placed on one half of wooden trays 1.2 m × 0.9 m × 10 cm deep. The trays have slatted, split cane bottoms and are divided into two sections by means of a movable partition. Twelve such trays are stacked so that the filled sections are above one another and the entire stack rests on an empty tray to allow better aeration. The top tray is covered with banana leaves and sacking. After twenty-four hours, a close fitting sack is slipped over the stack. They suggested that no handling was required until the stack was dismantled for drying after three days.

Factors Which Affect Fermentation

Duration

There are wide variations in this fermentation variable throughout the world's cocoa growing regions. Traditionally, the criollo cocoas have been fermented two to three days and forastero type cocoas have been fermented from five to eight days. We found six methods are used to determine when to stop fermentation and begin drying:

1. Time schedule
2. Bean sample cut, and the internal color used as a criterion
3. External color of beans
4. Smell of the fermenting mass
5. Fall in temperature
6. Plumping of the beans

Aeration

Early investigators of cocoa fermentation thought that beneficial changes were limited to oxidative reactions. However, currently the requirement for an initial anaerobic phase for successful fermentation is generally accepted. During the anaerobic phase, pulp sugars are converted to ethanol by yeasts. Ethanol is oxidized to acetic acid while oxygen is present during a short period after the beans are turned and after air penetrates the mass due to drainage of the pulp.

Batch Size

Due to the variation in the size of cocoa holdings and the fluctuation in yield during the harvest season, the amount of cocoa to be fermented at one time tends to vary dramatically. This influences the method of fermentation chosen as has been discussed. The mass of cocoa which is fermenting influences the degree of aeration and, therefore, to some extent, the amount of heat produced by the microorganisms. The increased aeration at the surface of a large fermenting mass results in a more rapid rise in temperature than occurs in the center of the mass. In very small amounts of cocoa, the surface aeration is proportionally increased to a critical point where air penetration is nearly complete. The increase in

temperature due to increased microbial activity tends to be negated by loss of heat to the surroundings.

Various authors have stated differing opinions regarding the minimum quantity of cocoa which can be properly fermented under natural conditions. These vary from 35 to 450 kg.

Mixing

Mixing or stirring the cocoa at various times during the fermentation process is necessary to introduce air into the mass, to achieve homogeneity, avoid clumping of the beans, and to discourage mold growth on the surface and/or corners of the fermenting heap or box. The mechanics of this operation require transfer of the beans from a heap fermentation to a new base of plantain leaves by hand or using a wooden shovel.

The timing of mixing during the fermentation varies. In some areas, the beans are turned after 24, 48, 96, and 144 hours and in others, the beans are turned daily. Mixing early in the fermentation period to increase aeration tends to favor ethanol production and discourage lactic acid formation.

Death of the Bean

During the fermentation period, the beans must be killed. Seed death is accompanied by the loss of cellular integrity and vacuolization. This permits the mixing of substrates and enzymes leading to the reactions which produce the precursors of chocolate flavor. During the first 48 hours of fermentation, ethanol and acetic acid are produced and accumulate to levels of about 3.5% and 0.4%, respectively. These levels to be sufficient to cause the death of the beans as measured by loss of germination capability and by diffusion of polyphenols from their storage vacuoles. In this study the influence of temperature on the rate of seed death was shown to be much less than that of ethanol and acetic acid.

Microbiology

Sources of Inoculum

The pulp is microbiologically sterile or nearly so, if healthy, undamaged pods are opened aseptically. Everything with which the pulp comes into direct or indirect contact after the pod is opened is a potential source of inoculum. A successful cocoa

fermentation requires a succession of microorganisms. However, it is thought that most of the organisms present are not essential. Some examined the phyllosphere microflora of several tropical plants including *Theobroma cacao*. Of the various yeasts isolated from cocoa leaves, only one species has been isolated as a component of the cocoa fermentation microflora: *Candida parapsilosis*. We found *C. guilliermondii* as a component of the phyllosphere of *Malpighia coccigera* L. and *Ixora coccinea* L. Some yeasts such as *C. krusei*, *Torulopsis candida*, and *Pichia membranaefaciens* were present from flower to ripe fruit and are found in large numbers in fermenting cocoa. Others such as *Saccharomyces* spp. are only found on ripe pods.

Yeasts were found in the tissues of the pods and may have infected the pod by invading the gynaecium during pollination. This explanation is probably true for yeasts found in unripe fruits which were *Torulopsis* sp. X. *Pichia pastoris*, and *P. membranaefaciens*, *Saccharomyces* sp. was only found in ripe pod tissue indicating another route of infection. Pulp infection percentages were quite variable between plantations which may be another variable in fermentation. Yeast numbers were one to a few hundred per gram of pulp and less in the walls and increased with pod ripeness.

Table 22.2 : Microorganisms Isolated from the Fruit Flies *Drosophila melanogaster* at Centeno Estate

Microorganism	Flies			
	1	2	3	4
<i>Acetobacter aceti</i>	+	+	+	+
<i>A. roseus</i>	+	—	+	—
<i>A. suboxydans</i>	—	+	—	+
<i>Arthrobacter simplex</i>	—	—	+	—
<i>Axotomonas insolita</i>	—	—	—	+
<i>Bacillus cereus</i>	—	—	+	+
<i>B. licheniformis</i>	—	+	—	—
<i>B. pumilus</i>	—	+	—	—
<i>B. stearothermophilus</i>	+	—	+	+
<i>Lactobacillus fermenti</i>	+	+	—	+
<i>Propionibacterium freudenreichii</i>	—	—	+	—
<i>P. shermanii</i>	—	—	+	—
<i>Streptococcus thermophilus</i>	+	—	—	—
Yeasts	—	+	+	+

+ Present, — absent

Some points out that *Drosophila*, ants, and mucoid flies are important in transferring yeasts and bacteria from one substrate to another. Microbes are important food sources of these insects.

Table 22.2 shows the organisms which isolated from four specimens of the fruit fly *Drosophila melanogaster*

Yeasts

Yeasts form a dominant group during the first 1-2 days of fermentation.

Table 22.3 : Yeasts Isolated from Cocoa Fermentations

Yeasts	Yeasts
<i>Candida</i> sp.	<i>P.memberanaefaciens</i>
<i>C.cutenulata</i>	<i>Rhodotorula</i> sp.
<i>C.krusei</i>	<i>Saccharomyces</i> sp.
<i>C.mycoderma</i> ^{a)}	<i>S.carlsbergensis</i> ^{b)}
<i>C.parapsilosis</i>	<i>S.cerevisiae</i>
<i>C.zeylanoides</i>	<i>S.cerevisiae</i> var. <i>ellipsoideus</i>
<i>Debaryomyces</i>	<i>S.chevalieri</i>
<i>Endomycopsis javanensis</i>	<i>S.rosei</i>
<i>Geotrichum candidum</i>	<i>Schizosaccharomyces</i> sp.
<i>Hanseniaspora</i> sp.	<i>S.pombe</i>
<i>Hansenula</i> sp.	<i>Trichosporon cutaneum</i>
<i>H. anomala</i>	<i>T.pulluans</i>
<i>Kloeckera</i> sp.	<i>Torulopsis</i> sp.
<i>K.apiculata</i>	<i>T.candida</i>
<i>Pichia</i> sp.	<i>T.castellii</i>
<i>P.farinosa</i>	<i>T.holmii</i>
<i>P.fermentans</i>	<i>T.rosei</i>
	Others

The wide variety of conditions present in a cocoa fermentation with respect to aeration pH, ethanol concentration, and substrate provide niches for several yeast species in each fermentation. The results of several investigations into the nature of the yeast flora of cocoa fermentation are listed below. (Table 22.3)

Many yeasts oxidize ethanol if sufficient oxygen is available. It also points out the fact that giving an isolate a Latin binomial is not

sufficient to describe metabolism. Many species are variable or strain specific for the assimilation of various carbon sources, growth temperatures, and other important physiological parameters.

Lactic Acid Bacteria

Lactic acid bacteria are a heterogeneous group that are characterized by a fermentation resulting in 50% or more lactic acid. Lactic acid bacteria are generally favored by a low oxygen concentration or if oxygen is present, a high concentration of carbon dioxide. This condition is found in the collapsed pulp as the yeasts are declining in numbers.

Lactic acid bacteria of the genus *Lactobacillus* are divided into homofermentative or those that produce lactic acid as the primary fermentation product and the heterofermentative which produce several products with lactic acid making up 50% or more of the total. Both groups are found in cocoa fermentations (Table 22.4)

Acetic Acid Bacteria

In portions of the mass that are well aerated, usually after the decline of yeast or lactic acid bacteria populations. The acetic acid bacteria become the dominant organisms. The genus *Acetobacter* is strictly aerobic and most strains can use hexoses and glycerol. At pH values from 7.0-4.5, ethanol is oxidized to acetic acid. Acetic and lactic acids can be oxidized to carbon dioxide and water. The optimum growth temperature is about 30°C with a range of 5-42 °C. The pH optimum is 5.4-6.3 with a range from 4.0-7.0.

Other Bacteria

A number of other bacterial species have been found in cocoa fermentations.

The importance of any given species is difficult to judge. *Leuconostoc* spp. may play a role similar to the *Lactobacillus* spp. while the *Micrococcus* spp. may contribute to the reduction in acidity by oxidizing acetic and lactic acids.

One species that has been isolated frequently and could be important is *Zymomonas mobilis*. Its optimum growth temperature is 30°C. It grows well in high sugar environments with a pH between 3.7 and 8.0. It is capable of fermenting glucose, fructose,

and sucrose to ethanol and CO₂ with traces of lactic acid. If cultures are aerated, ethanol can be oxidized to acetic acid. Although it is not an end product of metabolism, acetaldehyde may accumulate in the medium. In cocoa fermentations or portions of fermentations with a restricted oxygen supply, acetaldehyde and ethanol may kill the beans rather than acetic acid.

Table 22.4 : Some physiological Properties of Selected lactobacilli

<i>Species</i>	<i>Products of Glucose Chief Product</i>	<i>Fermenta- tion Minor Products</i>	<i>Growth Temperature</i>
<i>Homofermentative</i>			
<i>Lactobacillus lactis</i>	D(-)-Lactic acid		45°C+, no growth at 20°C
<i>L.bulgaricus</i>	D(-)-Lactic		
<i>L.acidophilus</i>	DL-Lactic acid		
<i>L.casei</i> subsp, <i>casei</i>	L(+)-Lactic acid	Acetate	
<i>L.plantarum</i>	DL-Lactic acid	Acetate	Growth at 15°C; optimum 30-35°C, no growth at 45°C
<i>Heterofermentative</i>			
<i>L.fermentum</i>	DL-Lactic acid	Acetate, ethanol	No growth at 15 °C; growth at 45°C

*Actinomyce*te growth is also considered to be a defect and it is considered to be the cause of at least some cases of musty odors and tastes.

Fungi Other Than Yeast

Filamentous fungi normally found in cocoa fermentations are considered to be a defect and steps are taken to suppress their growth. Because they are strictly aerobic organisms, they are largely restricted to the outer surface of the fermenting or drying cocoa mass. Because many of these fungi are xeric, their growth can continue until the moisture content of the content of the cocoa drops below 7% This is especially true for sun-dried cocoa.

Enzymology

Enzymes - General

Enzymes of cocoa autolysis and polyphenol oxidase have been studied for more than 75 years but the level of activity has never

been intense. Much of the early work was inadequate and several technical difficulties are presented by working with tissues with high polyphenol concentrations. Adequate techniques of polyphenol complexing and rapid separation of proteins from small molecules have only been developed in the last 20 years and still leave room for considerable improvement when used at the high polyphenol concentrations found in cocoa beans.

A number of enzymes have been reported from cocoa beans. The proof that the reported enzymes actually exist in cocoa beans is often poor, however, and reasonably good work has only been done on three classes of enzymes: anthocyanin glycosidases, polyphenol oxidases, and proteases. Table 22.5 lists enzymes reported from unfermented cocoa beans.

Table 22.5 : Reported Cocoa Bean Enzymes

Enzyme	Source				
	a)	b)	c)	d)	e)
β -Fructofuranosidase(invertase)	-	+			
β -Fructosidase(inulase)	-				
α -Glucosidase(maltase)	-	-			
α -Glucosidase(trehalase)	-	-			
α -Galactosidase(melibiose)		-			
α -Galactosidase(raffinase)	-				
β -Glucosidase(emulsin)	+	+			
β -Galactosidase(lactase)		-			
β -Galactosidase(anthocyanase)				+	+
α -Amylase(diastase)	-	+			+
Pectinesterase					+
Polygalacturonidase		-			+
Cellulose		-			+
Proteinase	+	-		+	-
Asparaginase			+		
Glycerophosphatase		+			
Phytase		+			
Lipase	-	+			
Polyphenol oxidase	+	+	+	+	+
Monophenol oxidase			+		
Peroxidase		+	+		+
Catalase		+	+		+
Asorbic acid oxidase			+		
α -Amino acid oxidase			-		

Glycosidases

The degree of fermentation of cocoa is often estimated by the color of the cocoa bean. Slate colored or blue-gray beans have not fermented at all. They have only been dried and the hydrophilic compartmentation is at least partially intact. We suggested that the slight blueing of the anthocyanin pigment is due to the liberation of ammonia from amino acids by the action of amino acid oxidases.

A deep reddish purple color is associated with reduced tissue pH and death and precedes significant glycosidase activity. The glycosidase splits the anthocyanin into sugar and anthocyanidin. The resulting change in the electronic structure of the chromophore results in a bleached violet color of the tissues under anaerobic conditions. The action of polyphenol oxidase in the latter stages of fermentation and during drying results in a brown color.

The pigments in unfermented cocoa beans have been identified as 3- β -D galactosidyl cyanidin and 3- α -L-arabinosidyl cyanidin

It is not known if one enzyme attacks both compounds or not but it is more likely that there is an enzyme that attacks α -linkages and an enzyme that attacks β -linkages.

All of the glycosidase work has been done with crude acetone powders.

This procedure requires drying and defatting the beans with petroleum ether and separation of white and purple "cells" by differential sedimentation. The white "cells" are then washed in 50% aqueous acetone, 80% acetone and finally anhydrous acetone to remove all traces of polyphenols and water at 0 °C.

This yields a free flowing white powder.

The powder was activated by passing a paste of 50 g powder and 300 mL of barbital buffer, pH 8.0, through a triple roll mill. The paste was washed off the rolls in 1 liter of buffer, blended for three minutes and centrifuged. The supernatant pH was adjusted to 4.2 with 0.2 N HCl and the precipitate was filtered off. Two volumes of ethanol were added to the filtrate off. Two volumes of ethanol were added to the filtrate and the solution was cooled to 0 °C. The resultant precipitate was collected by centrifugation and dissolved in pH 4.2 citrate-phosphate buffer. This enzyme preparation contained one half of the original activity. The degree of purification and specific activity were not given.

The enzyme was assayed by placing 200 mg of enzyme powder in 10 mL of buffer in Thunberg tubes. One half mL aliquots of 1:1 aqueous ethanol was placed in the stopper. Tubes were then evacuated to prevent polyphenolase reactions. After evacuation, the tubes were heated to 45 °C. This temperature was found to be optimal for the reaction in whole beans. Tubes were then mixed by inversion and the reaction was run for a fixed time. The tubes were cooled, opened, and the reaction stopped by adding 10 mL of 0.2 N HCl. Later, the final volume was made up to 50 mL with ethanolic HCl. The suspensions were filtered and the pigment destruction measured by measuring light absorption. Boiled controls were used.

The pH optimum of the enzyme powder was between 3.8 and 4.5. The boiled control showed slight activity between pH 6.2 and 7.0. The enzyme activity was partially inhibited by an unidentified component of the pigment vacuoles. The mode of inhibition is not known. Pure cyanidin arabinoside was stoichiometrically hydrolyzed to arabinose and cyanidin. The enzyme incubated preparation was stable between pH 4.0 and 9.0 at 45 °C for 17 hours, provided it was stored in vacuo.

Proteases

Changes in the proportions of protein amino acids of the fermented cocoa bean indicate that one or more proteases are active during the anaerobic-hydrolytic stage of fermentation. DEWITT speculates that this is due to more rapid evaporation of water from the fermenting cocoa which would dry them below some critical water activity for protease action. Water activity was not measured, but fresh and dry cotyledon weights are given. Water contents calculated from these do not support DEWITT's speculations. The moisture percentages were calculated from DEWITT's data by one of the authors (GRP). Heat treatment of cocoa beans at 40, 45 and 50°C did increase the free amino nitrogen, but not as much as during fermentation.

QUESNEL (1970, 1971, 1972) studied the temperature and pH optima of proteolysis in both acetone powders and tissue fragments. Acetone powders prepared by the method described for glycosidase showed no activity and the activity could not be restored by bubbling hydrogen sulfide through the reaction mixture. If 0.1% thioglycolic acid was added to the acetone water and acetone extractants, a powder with proteolytic activity could be

produced. This is suggestive evidence that cocoa protease(s) contains an active sulfhydryl group(s).

Polyphenol Oxidase

Because of its importance in cocoa curing and its relative ease of preparation, handling, and assay, polyphenol oxidase is the most studied cocoa enzyme. The polyphenolase reaction is responsible for the oxidation of (-)-epicatechin to a quinone. This quinone is capable of complexing with itself or other quinones to produce a brown substance of poorly understood structure. The oxidized (-)-epicatechin is also responsible for the irreversible tanning of proteins in the cocoa beans. This must be responsible for inhibiting some enzymatic activities and might also reduce substrate availability to proteases. The reactions of the polyphenolases and their reaction products are important to cocoa fermentation in that this set of reactions is responsible for the production of brown pigment, removal of tannin astringency and tanning of proteins. Protein tanning seems to be partially responsible for the absence of a "burnt feather" smell in roasted fermented cocoa.

Composition of Cocoa Beans and Chemical Changes During Fermentation

Sugars

Using paper chromatography, found evidence of D-fructose, D-glucose, D-galactose, sucrose, raffinose, stachyose, melibiose, mannotriose, and three unidentified sugars in commercial samples of roasted Caracas cocoa beans. In a later study, glycerol, meso-inositol, and the polysaccharides planteose, verbascotetraose and verbascose were additionally found in roasted Caracas cocoa beans. Roasting is generally considered to cause the reaction of sugars and amino acids leading to a decrease in the levels of free sugars. The compounds detected in these studies are the residual unreacted sugars, possibly the degradation products of some complex carbohydrates, or polymers formed during roasting, leading to an unrealistic picture of the flavor precursors of cocoa beans.

In freshly harvested cocoa beans, sucrose was the only sugar present in significant amounts. During fermentation, sucrose was hydrolyzed to glucose and fructose. The fructose to glucose ratio in

a sample of Trinidad cocoa beans sampled after three and seven days of fermentation was 2:1 and 1.2:1, respectively.

Polysaccharides

We reported the quantitative estimation of several polysaccharides. The analyses were performed on unfermented, dried beans. It is apparent that some fermentative changes must have occurred during the drying phase because no sucrose remained in the beans. The constituents found are listed in Table 22.6. No data are presented for the polysaccharide content of fermented beans.

The starch content ranged from 4.50% to 7.00% in samples from seven geographic regions of production. The average starch content was 5.30%. During a six day box fermentation the starch content gradually increased. At the start, 5.5% of the bean solids was starch and after fermentation, starch accounted for 6.5% of the solids content. The apparent increase in starch content was probably the result of the loss of soluble, non-starch solids. There is no evidence that the starch changes during fermentation.

Table 22.6 : Carbohydrates in Unfermented Cocoa Beans

Constituent	% in Dried Beans
Glucose	0.30
Sucrose	Nil
Starch	6.10
Pectins	2.25
Fiber	2.09
Cellulose	1.92
Pentosans	1.27
Mucilage and gums	0.38

Table 22.7 : Amino Acid Composition of Cocoa Protein Hydrolysate

Amino Acid	mg Nitrogen /Bean	Mol%
Aspartic acid	1.63	12.96
Glutamic acid	2.28	20.03
Glycine	1.43	6.41
Alanine	0.84	4.47
Valine	0.43	3.01
Leucine	0.67	5.24
Isoleucine	0.25	1.96
Serine	0.93	5.84
Threonine	0.72	5.13
Phenylalanine	0.68	6.71
Tyrosine	0.63	6.82
Cystine	0.07	1.00
Methionine	0.05	0.44
Proline	0.71	4.88
Tryptophan	0.18	1.10
Histidine	0.24	0.74
Lysine	1.36	5.94
Arginine	2.82	7.34
Ammonia	4.36	
Total	20.28 mg N/Bean	

Proteins

The content of various nitrogenous substances in unfermented criollo and forastero cocoa beans was determined. They found higher levels of total nitrogen in the forastero cocoa than in the criollo. They suggest that the higher level of protein in forastero is responsible for the increased fermentation time required by these varieties.

The nitrogen compounds in fresh cocoa beans were fractionated into ethanol soluble and insoluble groups. These groups were further separated. These data indicate that the fresh seed contains very small amounts of free amino acids and peptides. The amino acid composition of the cocoa protein was determined and is shown in Table 22.7.

Table 22.8 : Total Protein, Extractable Protein and Purity of Protein Preparations of Several Varieties of Cocoa Beans^{a)}

Cocoa Bean Variety	Total Protein ^{b)} mg g ⁻¹	Extractable mg g ^{-1c)}	Protein % ^{d)}	Protein Preparation Purity (%) ^{e)}
<i>Nonpigmented</i>				
Porcelana	206	66	32	31
Criollo	237	78	33	24
Catongo	268	89	33	44
<i>Pigmented</i>				
Nacional (sic)	268	186	70	53
UF 667	310	220	71	62
Pound 7	315	194	62	66

Table 22.9 : Protein Fractions of Six Varieties of Cocoa Beans (Determined from Their Solubility Characteristics)

Cocoa Bean Variety	Percentage Albumin	Distribution Globulin:	Prolamine	Glutelin
<i>Nonpigmented</i>				
Porcelana	33	19	11	37
Criollo	32	25	12	31
Catongo	37	19	13	30
<i>Pigmented</i>				
Nacional (sic)	51	25	12	12
UF 667	71	2	17	10
Pound 7	71	1	20	8

Lipids Cocoa butter is a relatively simple fat comprised primarily of palmitic, stearic, and oleic acids with small amounts of myristic, linoleic, linolenic, and arachidic acids.

Acids

ROHAN studied the volatile and non-volatile acids in commercial cocoa beans. Using a procedure employing ion exchange resins followed by distillation, they determined the volatile acids in cocoa beans from eight geographic origins. The principal volatile acid found was acetic acid. The mean content of free acetic acid in the samples studied was 0.30%. The total volatile acid content ranged from 0.33% to 1.14%. These data are shown in Table 22.10. The principal component of the non-volatile fraction was citric acid with smaller amounts of tartaric, lactic, and phosphoric acids. For quantification purposes, the titer of these acids in cocoa beans was expressed on an equivalent weight of 60. The content of the free nonvolatile acids was 1.26% and mean content of non-volatile acid salts was 1.05%. The total non-volatile acids ranged from 1.04% to 5.25%. The high value was observed only in one sample and was due to a high level of free citric acid. These data are also shown in Table 22.10.

Quantitative estimation of the individual non-volatile acids in commercial beans was performed. They found qualitatively seven organic acids and phosphoric acid in cocoa beans. The organic acids were gluconic, lactic, oxalic, succinic, malic, tartaric, and citric acids.

ROHAN studied the flavanoids and phenolic acids found in fermented cocoa. These included *o*-coumaric, caffeic, and chlorogenic acids. The phenolic acid fraction did not qualitatively change during fermentation. Therefore, the authors felt these compounds were not important to the development of chocolate flavor.

Table 22.10 Aromatic Acids Present in Cocoa

<i>Acid</i>	<i>Present in Cocoa</i>	<i>Unfer- mented Cocoa</i>
P-Hydroxybenzoic	+	+
Protocatechuic	+	—
Vanillic	+	+
Syringic	+	+
Phenylacetic	+	
<i>o</i> -Hydroxyphenylacetic	+	
<i>p</i> -Hydroxyphenylacetic	+	
<i>p</i> -Coumaric	+	+
Ferulic	+	+
Phloretic	+	
Aesculetin	+	

Eleven aromatic acids were found by in fermented cocoa. Five of these were present in unfermented cocoa. The compounds found are summarized in Table 22.10. Quantitative estimation of these compounds was not attempted. This class of compounds was studied to determine if they provided the honey-like or balsamic note in chocolate. Mixtures of the odorous compounds in this class did not reproduce the desired odor.

Recently, quantified many of the acids which were previously qualitatively described in cocoa (Table 22.11). Based on a limited number of samples, these compounds generally did not change dramatically with fermentation or upon roasting. However, protocatechuic acid increased during fermentation and also during roasting; vanillic acid content increased during roasting. Caffeic acid was destroyed during fermentation and during roasting. The significance of these compounds or their changes during post-harvest processing were not discussed.

Table 22.11 : Phenolic Acids and Neutrals Quantitated in Cocoa Beans ($\mu\text{g g}^{-1}$)

<i>Compound Detected</i>	<i>RF</i>	<i>Time (min)</i>	<i>Unfermented Unroasted</i>	<i>Fermented Unroasted</i>	<i>Fermented Unroasted</i>	<i>Fermented Roasted</i>
Phloroglucinol	0.78	3.1	3.89	3.79	2.90	3.19
Vanillic acid	0.57	3.7	0.79	0.30	0.79	3.17
Protocatechuic acid	0.21	4.9	3.38	11.8	12.8	23.4
Ferulic acid	0.55	8.9	3.23	2.40	2.04	2.16
2-Hydroxyphenyl acetic acid	0.38	11.3	0.57	0.38	-	-
p-Coumaric acid	0.41	13.7	0.53	1.52	0.81	-
Caffeic acid	0.24	16.7	11.13	3.47	1.41	0.14
Catechin	0.75	31.9	88.9	13.4	39.7	14.0

Pigments and Polyphenolics

The anthocyanin components of fresh cocoa beans were described as cyanidin-3 arabino-glucoside and an uncharacterized cyanidin glucoside. The anthocyanins were later purified, crystallized, and characterized. Original preparations were found to have been contaminated with colorless glucosides which confused the

structure determinations. The correct structure of the anthocyanins was determined to be 3-a-1-arabinosidyl cyanidin chloride and 3β-D-galactosidyl cyanidin chloride.

The flavan compounds of cocoa beans were also characterized and estimated. The major components were catechin type compounds and leucocyanidins. Table 22.12 summarizes the species identified and an estimate of their relative concentration. The minor catechin compounds were tentatively identified by cochromatography with known standards.

Table 22.12 : Major Fajor Flavan Compounds in Cooca Beans	
Compound	Concentration in Bean
Catechins	
(-)-Epicatechin	
(92% of fraction)	3.0%
(×)-Catechin	
(×)-Gallocatechin	
(-)-Epigallocatechin	
Leucocyanidins	2.5%

The leucocyanidin fraction was separated by paper chromatography into three spots which were not further characterized. Approximately 25% of the total polyphenols were not accounted for and assumed to be complex tannins.

During fermentation, the anthocyanins are degraded and they condense to insoluble tannins called phlobaphenes. The degradation results in a bleaching of color and is mediated by an enzymatic hydrolysis of the sugar moiety from the anthocyanin molecule. The aglycone resulting from the sugar hydrolysis (cyanidin) does not undergo further change under anaerobic conditions. However, this compound is oxidized during drying via polyphenol oxidase.

The catechins remain unchanged until after day two of the fermentation. These compounds decrease in concentration steadily through fermentation and drying. Sun-dried beans contain about 20% of their original concentration of catechins. The suggested routes of loss during fermentation have been via exudation and oxidation.

Simple leucocyanidins decrease in concentration after day two of the fermentation and are essentially absent by the end of fermentation. Complex leucocyanidins increase in concentration from day two to day four of fermentation after which the

concentration drops to about 90% of the original by the end of sun-drying.

Scientists estimated 0.9 to 1.7% theobromine in cocoa samples from several geographic locations. Purine Alkaloids decrease in the theobromine content of cocoa cotyledons during a seven day box fermentation and a corresponding concomitant increase in the theobromine content of the shell. They conclude the theobromine migrated from the nib to the shell.

Chemical Changes During Drying

The majority of the world's cocoa producers dry their cocoa by spreading it on trays or wooden platforms and depend on the sun to dry the cocoa. This is a very economical and satisfactory method if there are sufficient hours of sunshine when needed. On large estates with high production and in countries where much of the crop is harvested during the rainy season, some type of artificial drying is required. A large number of designs have been used throughout the world. The principal factor which affects the quality of cocoa beans is the rate of drying. It appears that a rapid drying rate using high air flow at moderate temperature (65°C) in the initial phase is preferred. This permits the surface moisture to evaporate without causing the cotyledon to shrink. This results in a testa permeable to oxygen. The final drying is performed at a higher temperature (80°C) using low air flow. With this system, browning of the beans is promoted and a minimum of flat beans with tightly adhering testas is produced. It appears that much research is needed to improve both the engineering aspects of drying and to discern the chemistry which occurs during this phase of cocoa processing.

Oxygen for the browning process is initially drawn from dissolved oxygen in the water between the cotyledon and testa of the bean. The evaporation of this water facilitates the access of gaseous oxygen to the cotyledon surface and, as a result, the rate of the oxidation process is accelerated. The rate increases until the bean reaches a moisture content of 20%. Thereafter, the rate decreases due to reduced enzymatic activity.

The final result of polyphenol oxidation is the production of polymeric brown pigments. These compounds are well recognized in a variety of products, but the detailed structural chemistry of these condensation reactions is rather obscure.

The bean testa is the main barrier of oxygen to the cotyledon. If the initial drying temperature is too high, the testa will adhere to the nib and limit oxygen transport. In this case, slightly purple beans will result because the catechins are not oxidized to quinone and therefore, the remaining anthocyanins are not oxidized. Slaty beans are the result of severe underfermentation in which the polyphenol containing vacuoles are not broken. In this case, glycosidase and polyphenol oxidase actions are prevented and conversion of the anthocyanin to anthocyanidin or oxidation of the polyphenols does not take place.

During fermentation, a reduction in the acetic acid content occurs. The major route of loss has been reported to be evaporation. Logically, metabolic oxidation of acetic acid by the microflora in the adhering pulp contributes to the reduction observed. The high acetic acid contents of some artificially dried cocoas may be due to drying at temperatures that inhibit biological oxidation.

5'-Guanosine Monophosphate

In Japan, konbu (kelp-like seaweed), katsubushi (dried bonito) and shiitake (a type of mushroom) have been habitually used as flavor enhancing materials. The flavor enhancing effects of konbu and katsubushi were found to be due to monosodium glutamate and the histidine salt of 5'-inosine monophosphate (IMP), respectively. It was found that 5'-guanosine monophosphate (GMP) was also a flavor enhancing component. Its action is more powerful than that of IMP and it is now produced in Japan at the rate of 1000 tone per annum. The methods for the production of GMP are as follows: (1) enzymatic hydrolysis of yeast ribonucleic acid (RNA); (2) chemical synthesis from 5-amino-4-imidazolecarboxamide riboside (AICAR); (3) phosphorylation of guanosine; (4) enzymatic conversion of 5'-xanthosine monophosphate (XMP) to GMP; and (5) direct fermentation from glucose.

Regulation of GMP Biosynthesis

Fundamental studies of metabolic regulation have provided an effective guide for the breeding of mutants with enhanced purine nucleotide, and nucleoside productivity. The biosynthetic pathways of purine nucleotides were elucidated by Buchanan employing a homogenate of pigeon liver (Fig. 23.1) and have been confirmed to operate in microorganisms belonging to the genera *Enterobacter* and *Bacillus*.

Three enzymes of the common pathway of AMP and GMP synthesis, phosphoribosylpyrophosphate amidotransferase, adenylosuccinate lyase and IMP transformylase, are repressed by end products such as adenosine and guanosine, while the intermediate derivatives such as inosine and xanthosine show only partial repression. IMP dehydrogenase, the enzyme specific for GMP synthesis, is repressed by guanosine, although xanthosine and inosine have no repressive effect.

Among the enzymes that are involved in the biosynthesis of nucleotides, nucleosides and bases, 5'-nucleotidase and nucleosidase have been shown to play important roles in nucleotide and nucleoside fermentation (Figure 23.2).

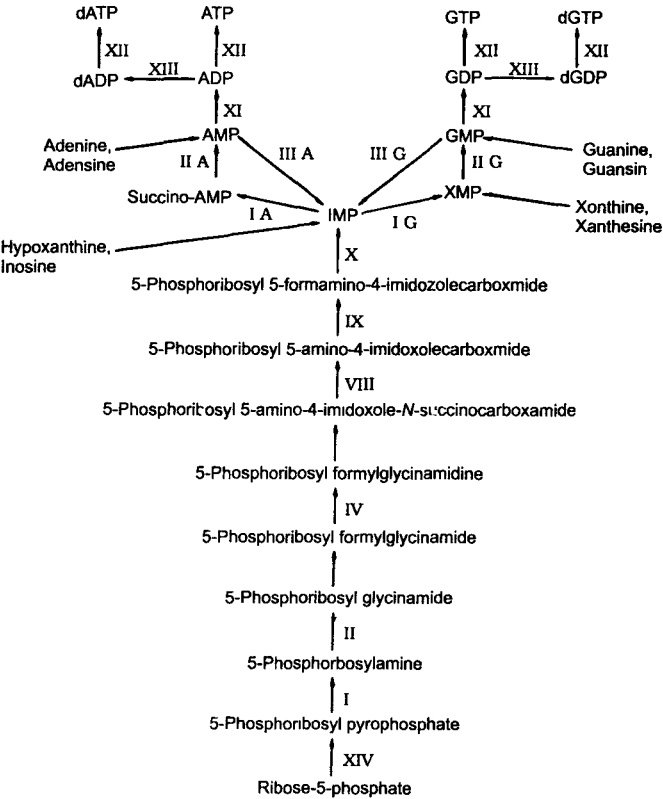


Fig. 23.1 : Biosynthetic pathway of purine nucleotides, I, 5-phosphoribosylpyrophosphae amidotransferase; IA, adenylosuccinate synthetase; IG, IMP aminase; II, 5-phosphoribosylglycinamide synthetase; IIA, VII, adenylosuccinate lyase; IIG, XMP reductase; IIA, AMP deaminase; IIG, GMP reductase; IV, 5-phosphoribosylformylglycinamide amidotransferase; IX, 5-phosphoribosyl-5-amino-4-imidazolecarboxmaide transformylase; X, IMP cyclohydrolase; XI, nucleotide kinase; XII, nucleoside diphosphate kinase; XIII, ribonucleotide reductatase; XIV, 5-phosphoribosylpyrophosphate synthetase.

Production of GMP

Enzymatic Hydrolysis of Yeast RNA

The production of 5'-nucleotides from yeast RNA involves the production of an RNA-hydrolyzing enzyme, hydrolysis of RNA by the enzyme, and separation and purification of 5'-nucleotides from the hydrolyzate.

A microbial enzyme which hydrolyzes yeast RNA to 5'-nucleotides was first obtained from a water extract of a solid culture of *Penicillium citrinum* on wheat bran. After extensive studies, found that similar enzymes are distributed in the culture broth of a variety of microorganisms belonging to the Ascomycetes, Fungi Imperfecti, actinomycetes and bacteria. Strain improvement was carried out to enhance the production of endonuclease, exonuclease and AMP deaminase, and to reduce that of 5'-nucleotidase and alkaline phosphatase. The enzymes now used industrially are obtained from cultures of *Penicillium citrinum* and *Streptomyces aureus*.

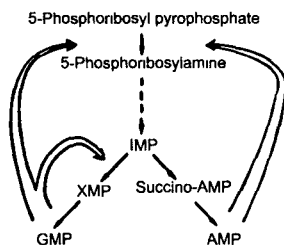


Fig. 23.2 : Regulation of GMP biosynthesis in *Bacillus subtilis*: ← inhibition and repression.

Yeast cells, the source of RNA, are cultivated in a medium composed of waste from the sulfite pulp process. The RNA content in the yeasts varies between 10-15% of the dry cell weight. RNA is extracted from the cells with dilute alkaline NaCl solution and precipitated by acidification or the addition of an organic solvent to the extracted solution. With heat-treated yeast cells, the process of RNA extraction is omitted, and the cells are subjected to the enzyme reaction.

Enzyme solutions extracted from solid cultures, contain many enzymes that hydrolyze RNA, nucleotides and nucleosides. The increased the yield of 5'-nucleotides by up to 90% of the original RNA content by hydrolyzing the RNA at 65 C and pH 5.0. The heat-labile phosphomonoesterase was shown to be inactivated.

Chemical Synthesis from AICAR

AICAR is produced by a fermentative process and separated from culture liquid by adsorption on a cation exchange resin, followed by elution, concentration and drying. The process for synthesizing GMP from AICAR is shown in Fig. 23.3. AICAR is allowed to react with sodium ethylxanthate, giving 2-mercaptinosine. The 2-mercapto derivative is then oxidized to give inosine-2- sulfonic acid and then aminated by ammonia to produce guanosine. Guanosine is phosphorylated with phosphoryl chloride to give 5'-GMP.

To produce sufficient quantities of AICAR the inhibitors of regulation of purine synthesis must be overcome. The main requirements for obtaining AICAR-producing mutants are as follows: (1) purine synthesizing capacity of the mutants should be high; (2) the mutants should lack AICARP formyltransferase, which is responsible for the conversion of AICARP to FAICARP; (3) the mutants should be free from feedback inhibition and repression of biosynthetic enzymes, especially of PRPP amidotransferase by intracellular purine nucleotides; and (4) the mutants should be deficient in AICAR hydrolyzing activity. An excellent producer of AICAR, *Bacillus megaterium* No. 335, was obtained this strain satisfied all of the above conditions and excreted 16 g l^{-1} of glucose.

In AICAR fermentation using a spore-forming *Bacillus megaterium*. The yield is markedly influenced by the degree of spore formation. Since only vegetative cells form AICAR, it is essential to restrict the spore formation for sufficient production of AICAR. The vegetative cells do not form spores when cell respiration is restricted ($R_{at}/K_{r_m} < 1.00$); however, AICAR accumulation is seriously depressed in oxygen-starved culture medium. On the other hand, when oxygen is in excess ($R_{at}/K_{r_m} < 1.0$), a large amount of spores appear and AICAR production is substantially reduced. Therefore, two incompatible factors must be satisfied in a culture; one is sufficient oxygen supply to ensure AICAR biosynthesis, and the other is insufficient oxygen supply to restrict spore formation. It is only during a period 8-12 after the beginning of the culture that spore formation is absolutely influenced by oxygen. When cell respiration is satisfied during this period, a large number of spores are formed during the stationary phase of cell growth. In contrast, when cell respiration is not satisfied at this time, spores are not formed at the stationary phase. Therefore, oxygen supply must be

controlled at a level between 0.85 and 1.0 R_{ab}/K_{rm} during the critical period, and 1.0 throughout the other periods of fermentation. Under these conditions, satisfactory accumulation of AICAR is observed (Figure 23.4).

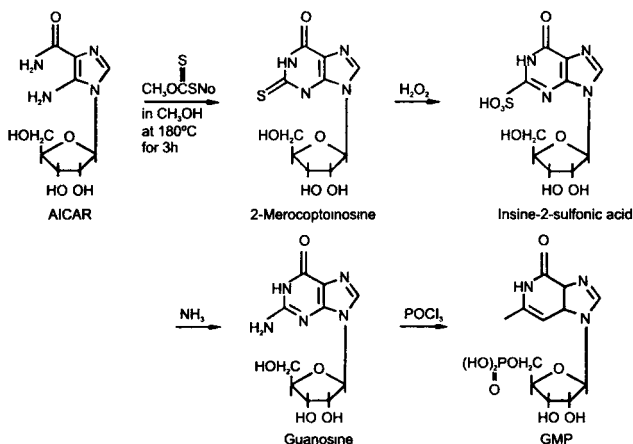


Fig. 23.3 : Synthesis of GMP from AICAR.

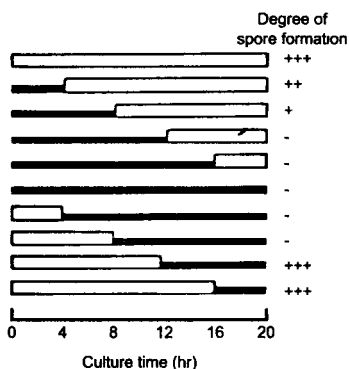


Fig. 23.4 : Effect of oxygen tension on spore formation : sufficient oxygen supply (dissolved oxygen above critical value for respiration); insufficient oxygen supply (dissolved oxygen recorded as zero by the membrane coated oxygen electrode).

Phosphorylation of Guanosine

Guanosine an intermediate in the chemical synthesis of 5'-GMP from AICAR (Figure 23.3). was produced from glucose by mutants

of *Bacillus subtilis*, and chemically phosphorylated to form 5'-GMP for industrial production.

In order to derive guanosine-producing strains, it is necessary to satisfy the following conditions based on the regulatory mechanisms in purine nucleotide biosynthesis: (1) adenylosuccinate synthetase deficiency; (2) GMP reductase deficiency; (3) deficiency in purine nucleotide hydrolyzing activity; and (4) enzymes of the GMP biosynthetic pathway, especially phosphoribosylprophosphate amidotransferase, IMP dehydrogenase and GMP synthetase being free from regulation.

The best producer, MG-1, was derived from an inosine- and guanosine-producing strain of *Bacillus subtilis*, which was a histidine auxotroph, and deficient in adenylosuccinate synthetase, GMP reductase and nucleosidase. MG-1 was resistant to methionine sulfoxide, psicofuranine and decoynine, and produced 16 gl^{-1} of guanosine from 80 gl^{-1} of glucose. In the mutants resistant to methionine sulfoxide, which is a glutamine analogue, the activity of 5'-nucleotidase was greatly decreased and conversely the activity of IMP dehydrogenase was larger than that of the parent strain. Furthermore, in the psicofuranine and decoynine resistant mutants, GMP synthetase activity increased remarkably, and the repression and inhibition by GMP were completely removed. Consequently, guanosine was overproduced by GMP synthetic enzymes free from the feedback effect of GMP, and accumulated at a high yield in preference to inosine and xanthosine owing to the increased enzyme level of IMP dehydrogenase and GMP synthetase rather than 5'-nucleotidase (Table 23.1)

Table 23.1 : Derivation of Guanosine Producers

Strain	Phenotype	Productivity (gl^2)		
		IR	XR	GR
1411	ade ⁻ , his ⁻ , GMP reductase ⁻	11.0	0	5.5
14119	ade ⁻ , his ⁻ , GMP reductase ⁻ , methionine sulfoxide ⁻	4.8	0	9.6
AG169	ade ⁻ , his ⁻ , GMP reductase ⁻ , methionine sulfoxide ^{rr}	0	6.0	8.0
GP-1	ade ⁻ , his ⁻ , GMP reductase ⁻ , methionine sulfoxide ^{rr} , psicofuranine ^r	0	3.4	10.6
MG-1	ade ⁻ , his ⁻ , GMP reductase ⁻ , methionine sulfoxide ^{rr} , psicofuranine ^r , decoynine ^r	0	0	16.0

Microbial Conversion of XMP to GMP

To produce GMP, a mixed culture of two different mutants of *Brevibacterium ammoniasenes* was examined, one of which produces XMP from glucose, the other converting XMP into GMP. A maximum production of 2.5 g^l⁻¹ of GMP was obtained from 100 g^l⁻¹ of glucose by selecting suitable inoculum proportions of the two strains and by feeding glucose and urea during the culture.

Fermentative Accumulation of GMP from Glucose

Genetic breeding of strains producing GMP from glucose are summarized in Table 23.2. The permeability barrier of the cell membrane to GMP was the major problem. GMP though accumulated within the cells as a result of enhanced biosynthesis by mutation, did not permeate through the cell membrane, but is excreted in the form of guanosine.

Table 23.2 : Fermentative Production of GMP

<i>Microorganism</i>	<i>Yield (g^l⁻¹)</i>	<i>(%)</i>
<i>Bacillus subtilis</i> (ade. amino acid)	2.2	3
<i>Escherichia coli</i> (ade. nucleotilase ^{neak})	0.9	1.8
<i>Micrococcus glutamicus</i> (ade)	5.1	5.1
<i>Brevibacterium ammoniasenes</i> (ade)	4.6	4.6

Tea Manufacture

What is Tea?

Origins of Tea

Tea is one of the most widely consumed beverages in the world. Tea is also one of the oldest known prepared beverages in the world. The earliest use of tea was by the Chinese, and this is known by written records going back to the 27th century B.C. and oral histories that describe events going back many more centuries. Since the development of the most characteristic attributes of tea (i.e. the flavor and color of the beverage) is dependent on manipulation of the tea leaves to induce certain endogenous biochemical changes, it can be said that tea manufacture is one of the oldest biotechnologies developed and practiced by mankind.

The scientific record indicates that the tea plant [*Camellia sinensis* (L.) O.Kuntze: Family Theaceae] originated somewhere in Southeast Asia and probably in what is now Vietnam. Today tea is cultivated around the world in tropical and subtropical areas: the largest tea producing countries are China, India, Sri Lanka, USSR, Japan, Kenya, and Indonesia.

Types of Tea

Many different types of tea are marketed in the world today. Fundamentally, they are all derived from the same species of plant, namely the tea plant *Camellia sinensis*. The differences between finished tea products is due to (1) quantitative differences in composition and physical properties of the fresh green tea leaves which are determined by the genetics of the clones of tea used, the climate, the soil, and the horticultural practices used, and (2) the manufacturing process used. The three basic types of tea are green tea (no fermentation), oolong and pouchong teas (partially

fermented), and black tea (fully fermented); and the amount of fermentation (this process is explained below) involved in their manufacture is the primary cause of their differences.

Physical and Chemical Characteristics of Tea Leaves

Only the most tender portions of rapidly growing branches of tea plants are used to produce the best quality teas. Rather long succulent shoots can be used for green tea manufacture, but only the tips of shoots are suitable for the manufacture of the finest quality black teas. These requirements relate to the physical properties of the tea plant parts: Immature plant parts are less woody, and therefore more workable in the tea manufacturing process. This is particularly important in black tea manufacture where a fermentation process must be induced.

The chemical composition of tea shoot tips is summarized in Table 24.1. Of particular importance are (1) the catechins (I-IV in Fig. 24.1) which comprise about 80% of the polyphenolic compounds in these plant parts, (2) the enzyme catechol oxidase within the protein fraction, (3) the caffeine which contributes the stimulating effect of tea drinking, and (4) the volatile compounds. Besides being woodier and, hence less workable, older leaves have less catechol oxidase activity and lower levels of catechins with adverse effects on their potential for conversion to tea products. Needless to say, it is the chemical composition and the physical properties of tea shoot tips that renders them uniquely suitable for the manufacture of tea beverage products.

Table 24.1 : Approximate Composition of Fresh Tea Shoot Tips

<i>Constituent</i>	<i>% of Total Dry Weight</i>
Protein	15
Fiber	26
Pigments (esp. chlorophylls and carotenes)	2
Lipids	7
Caffeine	4
Polyphenols (mostly catechins I-IV)	30
Amino acids	4
Minerals	5
Carbohydrates	7
Volatile compounds	0.1

*Moisture content of fresh tea shoot tips is usually about seventy-eight percent of total fresh weight.

Black Tea Manufacture

Harvest of Tea Shoot Tips

Tea bushes are regularly pruned to about one meter height to encourage a continuous supply of fresh shoot tips resulting from regrowth. These shoot tips are normally picked by hand to ensure harvesting of only those shoot tips at the proper stage of development and with minimal physical damage to the tissues involved. This ensures maximum yields of raw material with optimal composition for the tea manufacturing process.

Withering

The harvested tea shoot tips are transported to a tea factory (usually within a few kilometers of the tea field) where they are caused to undergo a loss of moisture from about 78% (fresh weight basis) to about 68% to 55% (withered weight basis): This process is called withering, and it is carried out under conditions that prevent the temperature of the respiring tea leaf from rising above about 24°C. The withering process takes from four to eighteen hours with methods in common use.

The primary object of the withering process is to destroy the semipermeable properties of the cell membranes without causing breakdown of the tissues involved. This enables tea fermentation to be uniformly initiated in the tea shoot tips during the subsequent leaf maceration process. Biochemical changes that are reminiscent of senescence take place during withering that appear to be important in producing substrates for the fermentation process.

Tissue Maceration (Rolling)

After withering, the tea shoot tips are macerated in such a way as to cause the contents of the cells in the tissues involved to become mixed. In the tea literature, this process is usually called “rolling”. Machines used for this purpose are designed to roll the plant material around while the shoot tips are being macerated. This action also aerates the mass of tissues and causes some temperature increase.

The process of tissue maceration is interspersed with “ball breaking” and screening (also called “dhooling”) to remove pieces of plant material that have been sufficiently broken up.

Tissue maceration takes from 30 to 90 minutes, and it initiates the fermentation process.

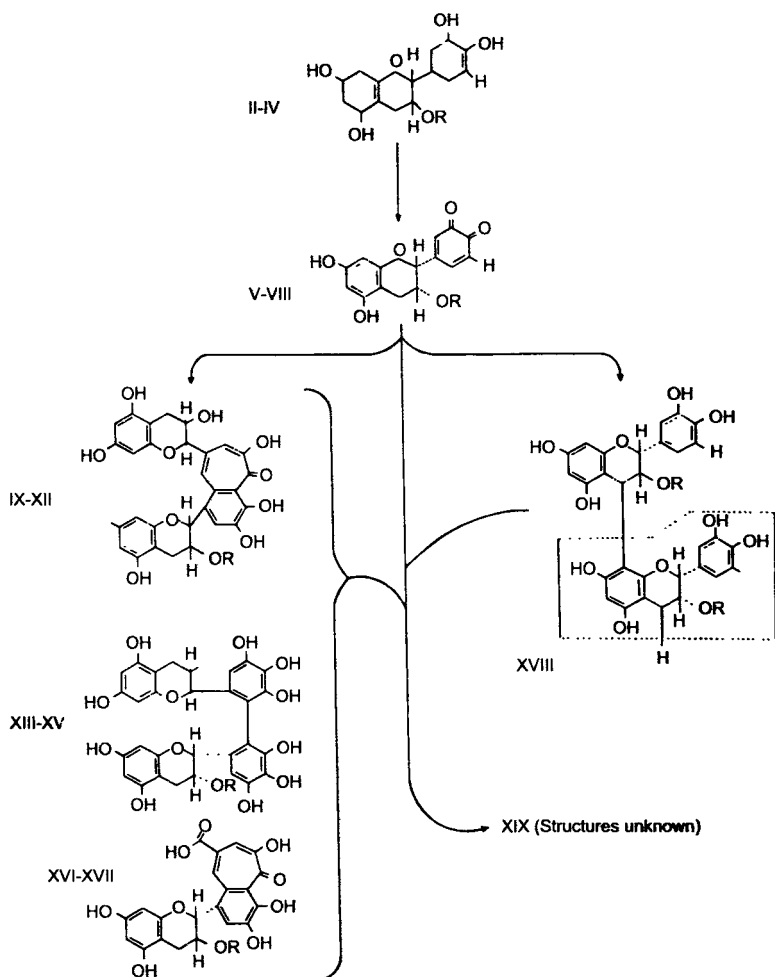
Fermentation

Tea fermentation is the oxidation of tea catechins mediated by the tea catechol oxidase enzyme and all the secondary reactions associated with this primary oxidation. These reactions are outlined in Fig. 24.1: All reactants except oxygen are endogenous to the tissues of the tea shoot tips. Under ideal conditions, the primary reactants of tea fermentation remain spatially separated until the tissue maceration step. Withering destroys the semipermeable properties of the cellular membranes allowing the cell contents to be come mixed under the action of the tissue maceration process. Once the catechins and the catechol oxidase are brought together in the presence of oxygen, tea fermentation begins.

As shown in Figure 24.1, the central reaction in tea fermentation is the oxidation of the tea catechins (I-IV) to orthoquinones (V-VIII).

Figure 24.1 : Pathway for oxidation of catechins during black tea fermentation.

- I. (–) Epicatechin: $R=R'=H$
- II. (–) Epicatechin: gallate; $R=\text{galloyl}$, $R'=H$.
- III. (–) Epigallocatechin: $R=H$; $R'=OH$
- IV. (–) Epigallocatechin gallate: $R=\text{galloyl}$; $R'=OH$.
- V-VIII. Oxidized catechins I-IV, respectively.
- IX. Theaflavin, $R=R'=H$.
- X. Theaflavin gallate A; $R=H$, $R'=\text{galloyl}$.
- XI. Theaflavin gallate B: $R=\text{galloyl}$; $R'=H$.
- XII. Theaflavin digallate; $R=R'=\text{galloyl}$.
- XIII. Bisflavanol A; $R=R'=\text{galloyl}$.
- XIV. Bisflavanol B: $R=\text{galloyl}$, $R'=H$.
- XV. Bisflavanol C: $R=R'=H$.
- XVI. Epitheaflavic acid; $R=H$.
- XVII. Gallated epitheaflavic acid ; $R=\text{galloyl}$.
- XVIII. Thearubigins (proanthocyanidin type); $R=\text{either } H \text{ or } OH$;
 $R'=\text{either galloyl or } H$.
- XIX. Thearubigins (polymeric catechins of unknown structures).



In turn, these orthoquinones may condense with one another to form theaflavins (IX-XII) (bright red color, fair solubility) or bisflavanols (XIII-XV) (colorless). They can act as oxidizing agents causing other substances present in the fermenting mass of the tea plant material to become oxidized. When gallic acid is so oxidized, condensation with an oxidized catechin will lead to the formation of epitheaflavic acids (XVI-XVII) (bright red color, excellent solubility). When theaflavins or epitheaflavic acids condense with oxidized catechins, polymeric thearubigins (XVIII-XIX) (dark brown

colored, fair to poor solubility) are formed. These reactions determine the color and the taste of the finished black tea product.

The aroma of black tea is also formed during the tea fermentation. (Aroma is of great importance because it is a most important component, together with taste, of tea flavor.) Black tea aroma is due to the effect of hundreds of volatile compounds present in minute amounts in black tea products. Some of these compounds are present in fresh green tea shoot tips, but the characteristic aroma of black tea is formed during fermentation. Experimental evidence indicates that the oxidized catechins (i.e. the orthoquinones V VIII) provide the major driving force for the reactions involved. Specifically, the oxidized catechins react with precursor molecules to produce the volatile compounds present in black tea aroma. Groups of compounds that have been shown to serve as precursors of important black tea aroma constituents in these reactions are (1) amino acids (2) carotenes, and unsaturated fatty acids.

Besides the critical role of catechin oxidation in the formation of black tea aroma, there is evidence that biosynthetic reactions are important in forming terpenoid compounds, and a metalloprotein may cause the formation of some black tea aroma constituents by direct catalysis of precursor transformation to volatile aroma constituents.

The fermentation process is normally carried out at ambient temperatures, and the process takes from one to five hours to complete. During this time the tea plant material changes color from green to coppery red, and the aroma changes from grassy to sweet and flavory.

Firing

Firing is the act of drying the macerated tea shoot tips after fermentation is completed. Drying is normally completed in about twenty minutes, using hot air driers operating with air inlet temperatures of 90°C, and exhaust temperatures of 52°C. The moisture content of the tea is normally reduced to 2-4% in this process.

During firing, catechol oxidase enzyme is inactivated but not before additional catechin oxidation takes place. Catechin condensation and interaction with other substances present in the

tea plant material is promoted by the firing process and produces the reddish colored substances (IX XII and XVI XIX) that are characteristic of black tea.

The chemical changes that take place during firing are not well understood, but they are most important to the development of the finished black tea. The coppery colored, greenish tasting fermented tea plant material going into the dryer is black colored, and has a pleasant flavory tea taste, when it leaves the dryer. Finally, the heat and the drying action drive off some of the volatile compounds with the result that a new balance is achieved between the more than three hundred black tea aroma constituents.

Grading and Storage

Tea is often winnowed to remove stalky material, and sieved to obtain different grades of tea based on particle size. This mechanical sieving does produce finished tea products with significant gradations of quality due to the fact that the most fragile portions of the tea shoot tips with their particular chemical composition tend to end up with the smallest (finest) fraction, and the leaf rib and stalk tend to end up with the coarsest (least flavory) fraction.

Tea will remain sound and full of flavor for more than a year if it is kept in a cool place and protected from moisture and oxygen. Storage under adverse conditions will lead to the development of undesirable flavors with consequent loss of value.

Green Tea Manufacture

Green tea manufacturing processes begin with a heat treatment to inactivate the enzymes, especially the catechol oxidase enzyme, present in the fresh green tea leaf material, which is used as raw material. This prevents the enzymic, oxidation of the tea flavanols and the preservation of the green color of the tea leaves throughout the manufacturing process. The chemical changes are subtle compared to those occurring during black tea manufacture, but they are important to the development of the characteristic flavor of green tea.

The traditional green tea manufacturing process used to make Japanese green tea, called Sen-cha, is summarized in Table 24.2. Many modifications of this process are used in the world, but they

all rely on a series of rolling and heating treatments to develop the qualities of the final product.

Table 24.2 : Summary of Green Tea (Sen-Cha Tea) Manufacturing Process Used in Japan.

<i>Step/ Process</i>	<i>Temp. (°C)</i>	<i>Time (min)</i>	<i>Result</i>
1. Steaming	100	0.7	Enzyme inactivated
2. Primary heating and rolling	70	30	48% Weight loss
3. Rolling	25	10	Leaf cells broken
4. Secondary rolling	50	20	68% Weight loss
5. Final rolling	160	50	Leaves twisted and dried
6. Drying	65	30	Moisture reduced to 4%
7. Refining and firing	110	20	Flavor modified

Oolong and Pouchong Tea Manufacture

Oolong and pouchong teas are characterized by being partially fermented. Oolong tea is about 50% fermented whereas pouchong tea is only about 30% fermented. These teas are made in China (esp. Taiwan) and their special flowery flavor appears to depend on local varieties of tea plants and the effect of the regional climate in determining the composition of the tea leaf material produced. Manufacture begins with a short solar withering period (up to 20 minutes) followed by indoor withering of 2 to 4 hours. During indoor withering the tea leaf material is turned over frequently causing bruising of the tea leaves which initiates some tea fermentation. Indoor withering is followed by pan firing which terminates the partially completed tea fermentation process. Rolling, firing, and finishing and firing again completes the process.

Flavored Teas

Some of the world's best known teas are made by adding flavoring materials to more basic teas. Jasmine tea is usually made by adding jasmine flowers to pouchong tea to enhance its flowery flavor. Earl Grey tea is made by adding bergamot oil to black tea. And there are many other flavored teas on the market with proprietary flavor concoctions added.

Instant Tea

Most commercial instant tea is made by extracting finished tea products and converting these extracts into soluble tea powders by

spray drying the extracts. The process is outlined in Fig. 24.2 . Most instant tea produced in the world is cold water soluble and is suitable for making iced tea.

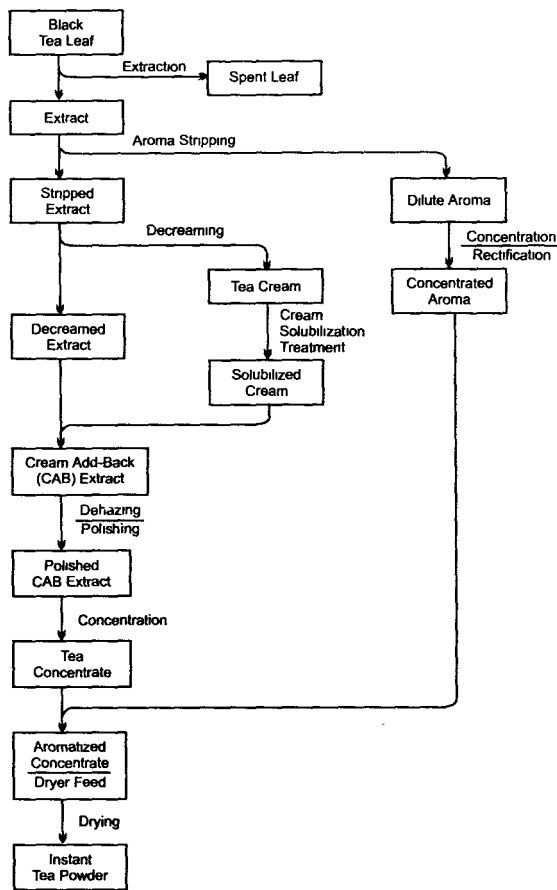


Fig. 24.2 : Outline of instant tea manufacturing process (cold water soluble).

Tea extracts may be concentrated and spray-dried directly to produce products that require hot water for redissolving. The requirement for hot water is due to insoluble complexes that form at room temperature between the caffeine and the polyphenolic compounds that are present in all tea extracts. Cold water soluble

instant tea products are manufactured by chilling the tea extract, separating the insoluble caffeine/tea polyphenol complex that forms, and treating this precipitate in such a way as to render it soluble. An alkaline-air mediated oxidation of the tea polyphenols is the most commonly used process, although an enzymic treatment using the enzyme tannase has been described.

Conclusion

Tea manufacture is one of the oldest biotechnologies that is today practiced on a wide scale around the world. It is the intention of this review to give an outline of the principles involved, but it should be recognized that details of the practices vary from place to place. And application of new developments in biotechnology can be expected to bring about more changes from traditional practices in the future.

Coffee Fermentation

Coffee has become everyone's drink although it used to have about it, not only a certain aristocratic but a religious atmosphere as well, with something ceremonial in its utilization. The stimulating effect of its aromatic beans has made the crop desirable and it is not surprising that legends have developed around it, although it is curious that there have been no proofs of its use in the Ancient World, nor, centuries later, in Greece, Rome and Byzantium.

One of the interesting things in the history of coffee is the perhaps somewhat legendary story of its introduction in Yemen by one of the culture-carrying invasions of the Sassanid conquerors. Other sources mention that the first use of coffee was found by the aborigenes of the African forests.

The earliest coffee beans for human consumption almost undoubtedly were chewed. A legend tells that a goat-herd found his animals dancing and cavorting after eating the fruits and branch-tips of certain bushes. He was curious and tested the same and he was stimulated to dance along with his goats in the Arabian hills. It so happened that a drowsy monk from below was passing by and admired the wakeful herd-boy, who told him his secret. The monk ate the fruits, the seeds and all, and quickly became reinvigorated, and he could pray longer without sleepiness. Anyhow, the use of coffee in Abyssinia was recorded in the 15th century and it was introduced later in Arabia. Advantage was taken of its action in preventing drowsiness in connection with the prolonged religious services of the muslims. The beverage then became popular in spite of the fact that strict muslim priests declared it to be an intoxicant and thus forbidden by the Koran.

In the early days of coffee commercialization, almost all of the worlds supply came from Yemen. Today Brazil and Colombia are

the largest coffee producing countries, although the first plantations existed in Indonesia in the 18th century. The beverage became known in England in the 17th century. The name coffee may originate from Arabian words such as kahwa. Coffee has given its name to establishments known first as coffee houses and later as cafés where now in most countries more beer than coffee is served.

The Coffee Plant

Coffee beans are produced by the genus *Coffea* belonging to the family of Rubiaceae. More than 40 species are known but only a few

are used in coffee production. The most important are: *C.arabica* (Linneaus) grown in Brazil, Central America, India and some African regions; *C. canephora* (Pierre ex Frehner) with its varieties *C. robusta* grown in Central America and Indonesia, *C. liberica* (Bull ex Hiern), and *C. excelsa*. Coffee plants are small-leaved shrubs, almost spiny in appearance, many of them hairy. Some have leaves that are dropped annually with the onset of the dry season, some are evergreens

holding their leaves for three or more years. The fruit is a fleshy berry about the size of a small cherry. As it ripens, it changes from green to a cherry-red color. Each fruit contains two seeds (beans) as shown in Fig. 25.1. The beans are embedded in a thin, parchement-like endocarp, surrounded by a yellowish pulp. This consists of an exocarp (or skin of the fruit) and a mesocarp made up of mucilaginous sub-stances. Directly inside the endocarp is the seed coat, which in Arabica is the "silver skin". The seed usually has a crease down the middle of its flattened side.

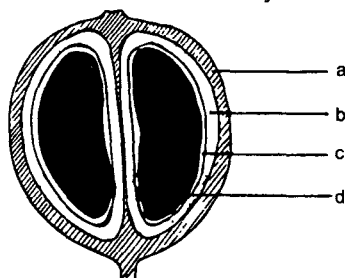


Fig. 25.1 : Drawing of the coffee fruit with (a) the exocarp or skin, (b) the mesocarp or mucilage layer, (c) the endocarp (hull), and (d) the seed (coffee bean) with the seed coat (silver skin).

Processing and Fermentation of the Coffee Fruit

The processing of coffee fruit consists mainly in the removal of the different layers surrounding the beans. Two methods are commonly used: a wet method and a dry method. A general flow sheet is given in Fig.25.2.

In the dry method, which is applied for most coffees of Brazil coffees, the fruits are first sundried during 2 to 3 weeks or with warm air. The dried fruits are then mechanically treated to remove the layers around the beans. The beans, known as green coffee, are screened according to size and marketed. Generally this method is said to be somewhat inferior to the wet methods with respect to final quality aspects of the beans. One advantage might be the production of less waste water. A classical wet method comprises the following steps: a) Depulping of the fruits by squeezing them through depulpers after wetting; b) natural fermentation of the depulped fruits; c) washing of the fermented beans; d) drying; e) dehulling and f) storage of the so-called "washed coffee". Storage may also occur before dehulling, especially for small production units, that rely on central dehulling plants. Normally the fermentation is carried out in open tanks (0.5-15 m³) where the depulped grains are submerged in water (wet fermentation) or kept in piles (dry fermentation) (Fig. 25.3.)

In the wet method waste waters are generated at several points and alternatives to reduce the pollution load have been suggested. In Fig.25.4 the different alternatives are situated in the general scheme of the wet method for coffee processing and fermentation. One of the interesting alternatives from a biotechnological point of view, seems to be a trickle bed



Fig. 25.2 : Simplified flow sheet of coffee processing by the dry or the wet method.

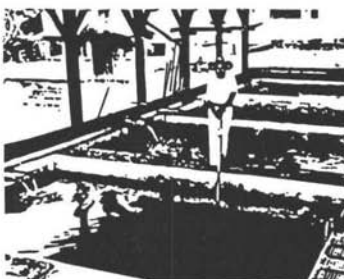


Fig.25.3 : Wet coffee fermentation showing a rectangular cement tank in which the depulped fruits are incubated during 24-96 h.

fermentation, where a broth rich in enzymes and microorganisms is recirculated through a bed of parched depulped coffee grains.

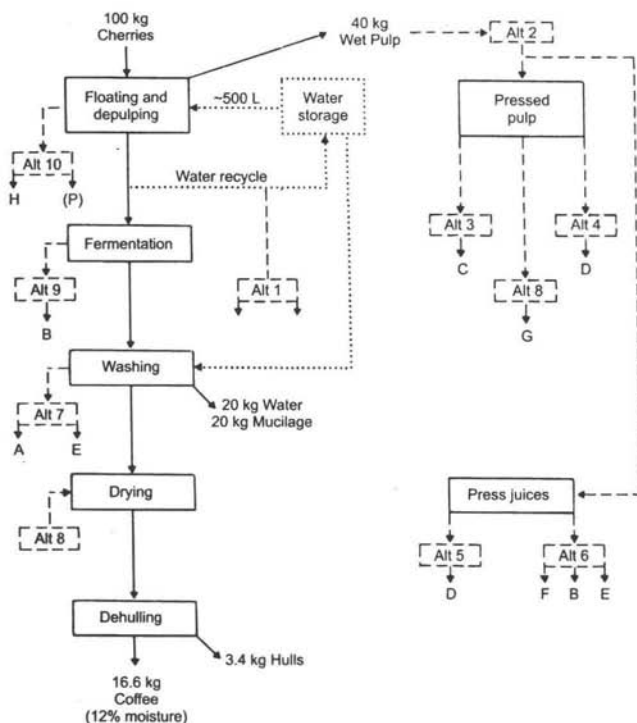


Fig. 25.4 : Flow sheet of wet coffee fermentation with different alternatives to reduce the pollution load or to increase the process efficiency. The alternatives are the following: 1. Recycling of water and/or water treatment to produce clean water (A) or obtain biomass (B). 2. Continuous pressing of pulp to obtain pulp juice and solid. 3. Decomposition of the pulp to produce fertilizer (C) or 4. Use of pulp as animal feed (D). 5. Production of pulp juice molasses and animal feed (D). 6. Utilization of pulp juice for fermentations to produce biomass (B), ethanol (F) or biogas (E). 7. Utilization of coffee wash waters to produce biogas (E) or clean water (A). 8. Use of biogas produced for drying and production of solid fuel (G). 9. Use of an alternative fermentation system (B). 10. Simultaneous depulping and demucilagination to produce coffee pulp juice (CPJ) or pectin (H).

Almost half of the world coffee production is processed by the wet method, comprising a natural fermentation. The latter requires

from 24 to 90 hours depending on climatic conditions, the degree of ripening of the coffee fruit and very likely the types of microorganisms present. A complete process may involve depulping during the night, fermentation lasting from 1 to 4 days, washing in the morning and then drying in the sun or with the help of hot air. *Coffea robusta* may require less fermentation time than *C.arabica*, although it should be recalled that *C. robusta* is generally grown in warmer climates than *C.arabica*. A pH of from 5 to 6 seems to be optimal, but a lowering generally occurs during fermentation, due to the production of organic acids. Coffee fermentation as contrasted to tea or cocoa fermentation, does not improve the final quality. It is a purely technical step. Coffee fermentation involves the digestion of the pulpy material of the mesocarp which frees the seeds. After fermentation the mesocarp layer can then be removed by washing with water. This removal is necessary to facilitate the further drying and the mechanical removal of the hulls. The pulpy material is mostly of pectic nature and fermentation involves pectolytic enzymes.

Alternative processes involve chemical or physical treatments of the coffee fruit, fermentations at higher than environmental temperature or the addition of enzymes. Some of these methods result in lower quality coffee beans and increase the price of a product that is subject to often unpredictable price fluctuations. For these reasons coffee fermentation in a natural way may remain the method of choice for many countries: it is simple.

Fermentation is stopped when, on inspection, the parchments no longer feel slimy. Fermentation does not improve coffee quality but it might induce some defects. When polluted waters are used in wetting or during fermentation, putrefactive processes can be initiated. Off-colors may also develop when the fermentation is not stopped in time, as a result of ethanol and/or acid production by the microflora. However, a certain acid character is sometimes desired.

Microorganisms Involved in Coffee Fermentation

Isolation and Characterization of Microorganisms from Zaire Coffee

The determination of total counts and total pectinolytic counts on the whole coffee fruits and on depulped fruits from several

Zairese regions indicated pectinolytic bacteria as being an important fraction of the microbial population. Small scale laboratory fermentations of fruits and depulped fruits showed always small but significant increases in total and pectinolytic activity as seen in Table 25.1. In general pectinolytic bacteria increased by a larger factor than total counts. Anaerobic pectinolytic bacteria were not detected. Bacteria isolated from media designed for growth of pectinolytic strains were mostly Enterobacteriaceae. *Erwinia dissolvens* was always detected. Less frequently were present: *Enterobacter liquefaciens*, *Enterobacter cloacae*, and *Bacillus cereus*. Yeasts that were present were not pectinolytic.

Table 25.1 : Changes in pH, microbial Population, and Pectinolytic Activity During Small Scale Coffee Fermentations (*Coffea robusta*)

Fermen- tation Time (h)	pH	Whole Cherries		pH	Depulped Cherries	
		Total Count ($\times 10^{-6}/g$)	Pectinolytic Activity ^a		Total Count	Pectinolytic Activity ^a
2	5.7	36	1.25	5.4	21	0.62
3	5.6	106	2.5	5.4	52	0.70
4	5.6	123	2.5	4.8	25	—
12	5.5	147	3.7	4.6	15	0.87
24	4.7	108	6.2	4.6	11	1.25
36	4.7	97	7.5	4.3	10	1.38
48	4.8	64	3.75	4.7	7	1.14

^a Expressed as pectinolytic microorganisms in counts $\times 10^6/g$

The Substrate for Fermentation Coffee Mesocarp

The mesocarp of the ripe coffee cherry as a colloidal gel, with 15% dry matter, of which 80% is pectin.

Pectin is generally characterized by the degree of esterification of the polygalacturonic acid chain (DE) and by the degree of polymerization (DP). When pectin was extracted from depulped cherries of *Coffea robusta* only about 30% of mesocarp dry weight was found to be pectin. The DE was around 71%. For a *C. arabica* from Guatemala a DE of 74% was found. The degree of polymerization can be determined by an enzymatic method using an *endo*-polygalacturonase from a *Pseudomonas* sp.

Mesocarp Degradation During Fermentation

As fermentation results in a loosening of the mesocarp, so that it can be washed away from the bean parchment, as the mesocarp contains 30% pectin and as pectinolytic bacteria are active during fermentation, it may seem logical to admit that pectin degradation is the main event during fermentation. Pectinolysis is an important phenomenon associated with many biological processes in plant material: elongation of cells, growth, ripening of fruits, etc. Pectic enzymes are also produced by many microorganisms and pectinolysis is important in plant deposits, digestion of plant material, retting processes. Pectic enzymes are classified in two main groups: de-esterifying enzymes (pectinesterases) and chain splitting enzymes (depolymerases). Fig. 25.5 indicates the main points of enzymatic attack.

PE is the point of attack by pectin methylesterase (pectase) which is a carboxyl ester hydrolase. Pectic acid and methanol are produced. Different methods exist to determine its activity. A continuous titration of free carboxyl groups is a usual method. *PEs* are formed by higher plants, fungi, some yeasts, and bacteria.

Depolymerases are numerous. Splitting of the polymer occurs by hydrolysis (hydrolases) or by β -elimination (lyases).

ENDO-PG splits pectic acid and is an *endo*-polygalacturonase. Products are galacturonic acid and digalacturonic acid. The activity is measured by the increase in reducing groups or the decrease in viscosity. They are formed in higher plants, fungi, bacteria, and some yeasts.

EXO-PG is an *exo*-polygalacturonase making galacturonic acid from pectic acid.

ENDO-PAL is an *endo*-pectate lyase with pectate or low methoxyl pectate as substrate and the unsaturated dimer of galacturonic acid as the main product. Activity is followed by the increase in optical density at 235 nm, due to increasing unsaturation. A molar extinction coefficient of 4600 is used. They are present in fungi and bacteria. EXO-PAL is the *exo*-counterpart of ENDO-PAL and forms the same product EXO-PAL are the only pectate hydrolyzing enzymes of bacteria.

ENDO-PL is an *endo*-pectin lyase, acting on high methoxyl pectin with the production of unsaturated oligogalacturonates. The

increase in optical density at 235 nm may be followed; using an extinction coefficient of 5500 activity may be determined. They are mostly fungal.

Are Plant Enzymes Involved in Coffee Fermentation?

Several parts of the coffee fruit have been examined by us for the presence of pectic enzymes. Enzymes were extracted. Hydrolases and lyases were not found in coffee pulp, mesocarp + endocarp + silver skin, coffee beans or press liquid obtained on depulping. However, pectin esterase was found in all parts. Using a titrimetric method the highest specific activity was found in the mesocarp (Table 25.2). The best activity was at pH 8.5.

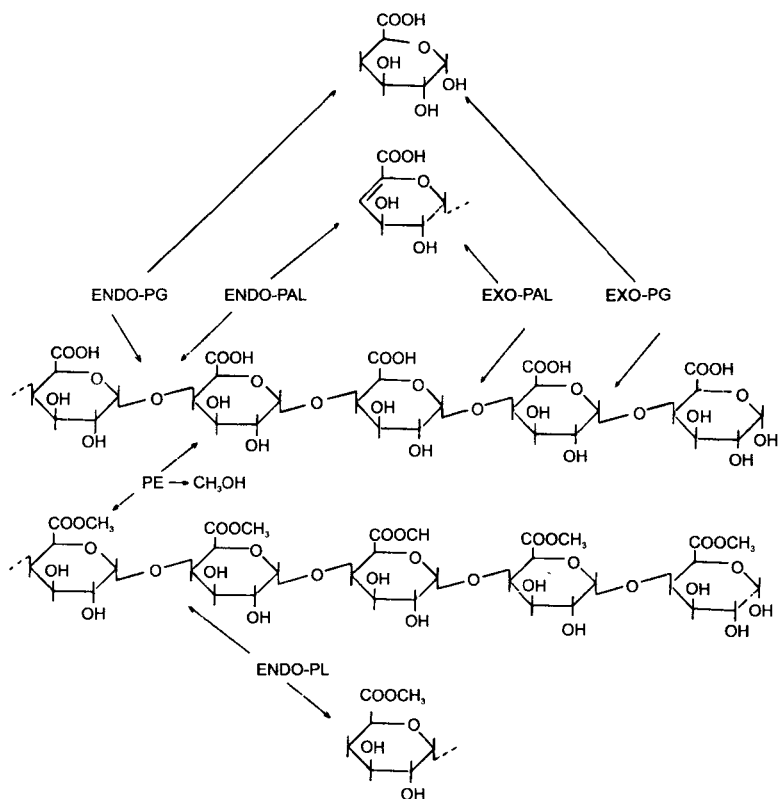


Fig. 25.5 : General scheme of possible enzymatic pectin breakdown.

Table 25.2 : Activity of Pectin Esterase from Parts of *coffea robusta* Fruit

Part of Fruit	Activity ^{a)}
Pulp	3.76
Mesocarp + endocarp + silver skin	5.10
Bean	2.19
Pulp press juice	3.02

^a Activity expressed in mL 0.01 N NaOH consumed after 10 min incubation and calculated per 100 mg of protein.

Are Microbial Enzymes Involved in Coffee Fermentation?

When this microorganism was grown in pectin media or in pectate media, only one enzyme could be detected. It was a lyase showing the following characteristics: temperature optimum at around 42°C, pH optimum at around 8.5 no requirement for calcium ions and thus resembling an *exo*-pectate lyase of *Erwinia aroideae*.

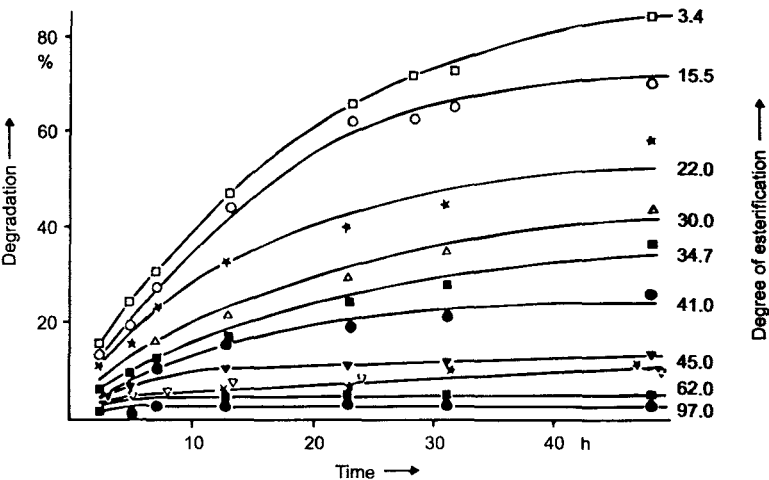


Figure 25.6 : Percentage of pectin degradation using pectins with increasing degree of esterification and the *exo*-pectate lyase from *Erwinia dissolvens*. -Degradation of pectin isolated from *Coffea robusta* mesocarp is shown by the line in between pectin with 62% DE and 45% DE. Break-down was measured by detection of unsaturated galacturonate.

In Fig 25.6 the formation of unsaturated digalacturonic acid from prepared plant pectins is compared with its formation from *Coffea robusta* pectin. Clearly the coffee pectin with its high degree of esterification is degraded. When depulped and HgCl_2 sterilized coffee fruit was inoculated with *Erwinia dissolvens*, mesocarp pectin was degraded (Fig 25.7). The last figure also shows pectin degradation in the mesocarp when natural fermentation occurs, when it is enriched with *E. dissolvens* or when it is enriched with the culture filtrate of an *E. dissolvens* culture. As shown most of the pectin is degraded during the first 8 hours. The highest final degradation (after 48 h) was observed when fermentation occurred in the presence of a culture filtrate of *E. dissolvens*, in the presence of EDTA. In this culture filtrate the highest pectate lyase activity was present. The pectin degradation was lowest with the pure culture. High blank values are due to a partial pectin solubilization. When the pectin contents of the fermentation media were examined, it was found that in the control experiment more pectin was present than in presence of microorganisms and enzymes, which of course solubilize the pectin with concomitant degradation. Such results clearly evoke the role of pectinolytic-bacteria during coffee fermentation.

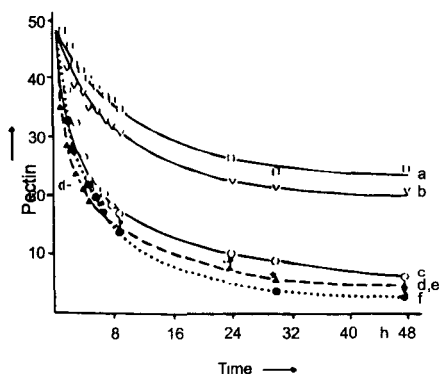


Fig. 25.7 : Residual amount of pectin in the mesocarp of *Coffea robusta* after fermentation. Line a: blank; b: with a pure culture of *Erwinia dissolvens*; c: natural fermentation; d: in the presence of a culture filtrate of *E. dissolvens* in the absence of EDTA; e: natural fermentation with inoculation with *E. dissolvens*, and f: in the presence of a culture filtrate of the bacterium in the presence of EDTA.

Conclusions

The microbial flora of coffee fruit (depulped or not) consists to a large part of Enterobacteriaceae. Some of these, especially *Erwinia* and *Enterobacter* species, are pectinolytic. Coffee mesocarp contains a high percentage of highly esterified pectin. Enzymes from the fruit may deesterify this pectin. Microbial enzymes may then depolymerize the pectate with a high degree of polymerization through a pectate lyase action. It is proven that *Erwinia dissolvens* produces such an enzyme and that the organism and the enzyme can be detected during coffee fermentation. The fact that other enzymes, such as hemicellulases, are also active cannot be excluded, as the mesocarp contains more non-pectin than pectin. Biotechnological alternatives for the fermentation process will certainly be looked into but it should be remembered that simplicity is one of the major advantages of the natural fermentation.

26

Fats and Oils

Commercial Oils and Fats

The market for oils and fats is a slowly expanding one, growing probably at a rate slightly faster than the increase in population. The demand for oils and fats is met largely from plant sources with animal fats and marine oils contributing less than 25% of the total production. The expansion of trade naturally puts pressure on the commodity and in the first instance the increased demand can be met by the simple expedient of growing more crops. The crop which contributes most significantly is the soybean which, although having only a 16% oil content, nevertheless contributes over 15 million tonnes of oil out of a total annual production of 60 million tonnes from all sources. There is, though, a natural limit to the extent to which the soybean can be grown. The two countries which are principally involved are the USA, which contributes 65% of the world's production, and Brazil, which contributes about 11% of the total. The uses of fats and oils are presented in Table 26.1. Approximately 30% of the total consumption goes into industrial products. The fatty acid composition of the major oils and fats is given in Table 26.2.

Oleaginous Microorganisms

Background and Definitions

Microorganisms have long been known to produce lipids and therefore to be potentially useful for the production of oils and fats. Such organisms may be termed oleaginous in keeping with the terminology used for oil-bearing plant seeds. For the most part, the oils produced by the oleaginous strains of eukaryotic microorganisms approximate to the oil produced by plants; that is they contain mainly C_{16} and C_{18} fatty acids esterified in the form of

triacylglycerols. The opportunities for a microbial oil displacing a conventional oil, such as soybean oil or palm oil, must however be considered remote. The economies of large-scale fermentations involving high technology would not seem to be able to compete against the low technology of agriculture. Thus costs of microbial oils must be considered to be several-fold more than plant oils. There could though be opportunities for the production of higher value-added commodities as well as for producing oils and fats from waste materials. The latter could be more a cost-effective

Table 26.1 : Uses of Fats and Oils

<i>Edible</i>	
Margarine	Soybean oil, groundnut oil,
Cooking fat	cottonseed oil, sunflower oil,
Cooking oils	rapeseed oil, sesame oil,
Salad oils/mayonnaise/table oils	palm oil, some fish oils,
Ice cream	olive oil, castor oil,
Confectionery	lard and tallow
Cosmetics, toiletries and pharmaceuticals	Coconut oil, palm kernel oil, castor oil
<i>Non-edible</i>	
Detergents and surfactants	Palm kernel, coconut oil
Soaps, metallic soaps, synthetic waxes	Palm oil
Paints and coatings	Linseed oil, tung oil, soybean oil, sunflower oil
Varnishes and lacquers	Linseed oil, tung oil
Inks	Various, mainly castor oil
Plastics and additives	Various, mainly soyabean oil
Lubricants and cutting oils	Castor oil, coconut oil
Wood dressings, polishes	Tung oil
Leather dressing	Fish oils
Metal industry	Palm oil and tallow
Agrichemicals, long chain quaternary compounds as herbicides, insecticides and fungicides	Various, mainly soybean oil
Evaporation retardants	Fatty alcohols from any source
Fabric softeners	Tallow

Table 26.2 : Fatty Acyl Composition of Commercial Plant Seed Oils and Animal Fats (from Procter and Gamble, 1979)

Commodity	below	Relative % of fatty acyls								20:0 and over
		12:0 and 14:0	16:0	16:1	18:0	18:1	18:2	18:3	Ho- 18:1	
Castor			2	—	1	6	3	—	875	—
Coconut	63	18	8.5	—	3	6	1	10.5	—	—
Corn			12	—	2.5	29	56	0.5	—	—
Cottonseed		1	24	1	2	17	55	0.3	—	—
Groundnut	—	—	115	—	2	52	27	—	—	—
Linseed			6	0.5	3.5	19	14	57	—	—
Olive	—		12	2	2	70	13	0.5	—	0.5
Palm		1	45	—	3.8	40	10	0.2	—	—
Palm kernel	56	18	8	—	2	14	2	—	—	—
Rapeseed ^a	—	—	5	—	2	63	20	9	—	1
Soybean	0.5	0.5	12	—	4	25	52	6	—	1
Sunflower	—	—	8	—	3	20	67.8	0.5	—	0.7
Tung	—	—	4	—	1	8	4	83 ^b	—	—
Lard	—	1.5	27	3	14	43.5	10.5	0.5	—	—
Tallow	—	3	25	3.5	21	43	4	0.5	—	—

^a Canadian (Canola or Canbra) rape (zero erucic). ^b 3 parts= elecostearic acid.

process than producing single cell protein to be sold as animal feed. There are also opportunities to develop microbial lipids in those countries which may have a surfeit of cheap fermentable substrates but are unable to grow the requisite plants and face difficulties with the necessary balance of payments with which to effect the necessary importation of these commodities.

A definition for what constitutes an oleaginous microorganism poses some difficulty. A pragmatic definition would suggest that a microorganism containing more than 20-25% oil could be deemed a suitable candidate for commercial consideration. However, this should not be taken so as to deny that organisms with less lipid may not also be useful but these organisms could hardly be classed as oil-bearing if their oil content was much less than, say, 15%.

A biochemical definition for yeasts (and probably for moulds and eukaryotic algae) can be offered, though this will not hold for bacteria. A correlation has been observed between the possession

of the enzyme ATP: citrate lyase and the ability of a yeast to accumulate more than 20% of its biomass as lipid. The significance of the enzyme is that it serves to produce the substrate for fatty acid biosynthesis, acetyl-CoA, from citrate:



Acetyl-CoA cannot be produced in the cytoplasm from pyruvate (this reaction proceeds in the mitochondria). Oleaginous yeasts and, as suggested above, probably other oleaginous eukaryotic microorganisms accumulate citrate in the mitochondria which is then transported into the cytoplasm and there cleaved by ATP:citrate lyase. Non-oleaginous organisms do not possess the citrate-cleaving enzyme and must rely on less effective means of producing acetyl-CoA in the cytoplasm.

Accumulation of Lipid

As lipid represents a reserve storage material, it is not unexpected to find that lipid accumulation is favoured by oleaginous microorganisms growing in a medium with a high carbon to nitrogen ratio. Usually a C:N ration of 50:1 is employed. In a batch culture, the organism grows until the nitrogen is consumed but thereafter it continues to take up the excess carbon and convert this to lipid. Thus a biphasic growth pattern can be envisaged.

With some of the slower growing moulds, the rate of lipid accumulation appears to coincide with the growth rate. Although this is probably fortuitous, the result is that the lipid content of the cells increases at the same rate as growth proceeds.

In continuous culture, lipid accumulation is achieved by growing oleaginous microorganisms under nitrogen-limiting conditions at a dilution rate (=specific growth rate) of about 30% of the maximum. The build-up of lipid is dependent upon the correct balance being achieved between growth rate and the specific rate of lipid biosynthesis so that the optimum amount of carbon can be diverted into lipid and the minimum into other cell components.

The efficiency of lipid accumulation in continuous culture is often the same as or better than in batch cultures where the same organism has been studied under both conditions. With *Candida* sp. 107, *Rhodotorula glutinis*, *R. gracilis* and *C. curvata*, lipid yields of 17-22% have been obtained under both conditions of growth. A

conversion of carbohydrate to lipid of 20% would appear near to a possible practical limit as the theoretical maximum is about 33 g triacylglycerol from 100 g glucose (Ratledge, 1982) assuming that all the carbon of the medium is converted into lipid without synthesis of any other cell component.

Bacteria

Triacylglycerols

Only a few species are known which produce appreciable amounts of extractable glycerol lipids. The mycobacteria-nocardia group of organisms are well known for their high lipid contents but these lipids are complex structures often in a bound form as part of the cell envelope structure. Some of these species do contain triacylglycerols but exploitation of them is not sensible as the coextraction of toxic or allergenic substances from the mycobacteria is highly likely.

The only bacterium which has been reported as producing significant amounts of triacylglycerol is *Arthrobacter*. This organism is unlike any other bacterium in that it can contain up to 80% of its biomass as lipid; this lipid, moreover, is predominantly composed of triacylglycerols and would thus seem an excellent candidate for commercial exploitation. The composition of the fatty acids of this organism is given in Table 26.3.

Poly- β -hydroxybutyrate

Many bacterial species produce the polymeric poly- β -hydroxybutyrate (PHB) as a reserve storage polymer. Although PHB is not a fatty acid-containing lipid (Fig. 26.1), it is nevertheless classified as a lipid in view of its solubility in chloroform and similar solvents. Like the lipids of the eukaryotic microorganisms, PHB is produced in increased quantities when nitrogen is exhausted from the medium. However, its synthesis also responds to the concentration of O_2 . At low partial pressures of oxygen the ability of the bacteria to reoxidation of $NADH$ becomes limiting. By producing PHB, the organism is able to achieve reoxidation of $NADH$, in much the same way as ethanol and butanol are produced as reduced fermentation products by other organisms. The structure of PHB is given in Fig. 26.1; its monomer, β -hydroxybutyrate, is synthesized from two acetyl-CoA units. The

degree of polymerization is between 4000 and 10 000 with the average molecular weight being 5×10^5 .

PHB is currently being considered for commercial exploitation by ICI Ltd. at Billingham, UK. It has been given the trade name of Biopol and is classed as a biodegradable thermoplastic. Its applications vary from acting as a substitute for plastics in roles where biodegradability would be an important attribute, to applications in the manufacture of hi-fi equipment where its piezoelectric properties would be useful. In the former case, the manufacture of surgical pins and sutures would be an important application.

Table 26.3 : Fatty Acyl composition of *Arthrobacter* AK19 (from Wayman *et al.*, 1984)

	<i>Time of growth (days)</i>		
	3	8	13
Total lipids, (% cell dry wt)	16.0	39.5	78.3
Fatty acyl composition of triacylglycerols (% w/w)			
14:0	2.7	3.2	3.1
15:0	2.8	2.2	1.9
16:0	36.1	23.9	30.1
16:1	12.0	13.2	14.4
br 17:0	< 0.3	< 0.3	0.6
17:0	4.4	1.4	2.5
17:1	7.3	8.4	7.0
br 18:0	< 0.3	< 0.3	0.4
18:0	7.8	5.1	5.4
18:1	23.9	35.0	29.8
br 19:0	1.4	1.7	2.7
Other	1.0	0.3	2.1

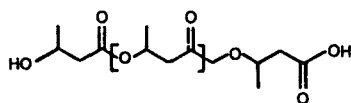


Fig. 26.1 : Structure of poly- β -hydroxybutyrate

The organism of choice for PHB production is *Alcaligenes eutrophus* which produces between 70% and 80% of its biomass as the polymer. The substrate currently used is glucose, though the possibilities either of being able to use another organism or of costs and thus open up new horizons for its applications.

Waxes

Waxes, although unusual amongst microorganisms, appear to be a common, though not major, lipid constituent of the *Acinetobacter*. Their composition is of a simple ester of a fatty acid with a fatty alcohol, $\text{CH}_3(\text{CH}_2)_x\text{C}(=\text{O})\text{OCH}_2(\text{CH}_2)_y\text{CH}_3$ where x and y are usually either 14 or 16, though shorter chain alcohols with $y=1$ to 3 have been reported. Unsaturation in both the alcohol and fatty acid components has been reported.

The amount of wax produced is usually small when these bacteria are grown on succinate or acetate (the bacteria do not grow on glucose). Typical figures indicate that the waxes constitute about 10-25% of the lipid, but this only represents about 2-3% of the cell biomass. However, growth on hydrocarbons and fatty alcohols increases the yield many-fold, with up to 15% of the cell biomass as waxes being recorded for *Acinetobacter* sp. HO1-N after growth on hexadecanol.

Table 26.4 : Comparison of the Wax Esters Produced by *Acinetobacter* sp. HO1-N Grown on Hydrocarbons and Those Found in Sperm Whale and Jojoba Oils

	Sperm whale Oil	Microbially produced wax esters	Jojoba oil
Carbon number of intact wax easters	28-40	32-40*	36-44
Acyl segments Carbon number	14-22	16-20*	16-24
Number of unsaturations	0.1	0.1-0.7	1
Alkoxy segments Carbon number	16-20	16-20*	18-24
Number of unsaturations	0.1	0.1-0.7	1
Predominant sites of unsaturation	7, 9 and 11	7 and 9	9
Yield of waxes (%cell dry wt)	-	2.6-8.5	-

Although the early studies with this strain did not reveal the occurrence of unsaturated waxes. These have subsequently been identified by capillary gas chromatography. These waxes are thus

similar to the commercially important sperm whale oil and jojoba oils (Table 26.4) though the percentage of diunsaturated wax is only about 10% of the total wax when hexadecane is used as substrate. Growth on eicosane (C_{20}) 17 °C, however, produces 6% of the biomass as waxes with 71% being diunsaturated.

Other lipids

Glycolipids with surfactant properties have been reported to be produced by a number of bacteria. Some of these compounds are more correctly classed as non-lipids for, although they do contain some lipid moiety, they are not soluble to any extent in organic solvents. Most of these glycolipids are produced in significantly increased amounts when the organisms are grown on hydrocarbons.

Algae

The oil content of microalgae has been reported to be as 70% with *Chlorella pyrenoidosa*. Although even higher claims have been made, more reasonable ones would put this maximum, no higher than 50%. The major constraints to development of algae are availability of land, water, sunlight and a warm ambient temperature.

Microalgae cultures should reasonably be expected to be able to achieve productivities of $15\text{--}25\text{ gm}^{-2}\text{d}^{-1}$, which is less than 10% of the theoretical maximum. Given an oil content of 50% of the biomass, yields of oil could be $25\text{ tonne ha}^{-1}\text{ y}^{-1}$ which is significantly higher than any plant oilseed crop. Dubinsky calculated similarly that an annual oil yield per hectare by algal culture should realize 22.8 tonnes. However this yield is not high enough to be economically viable in view of the large capital investment costs that would be required. The suggested solution would be to couple algal culture to sewage treatment which would then make the entire process economically feasible. This could also apply to algal culture in more temperate climates.

Although there are many algae which will produce more than 20% of their biomass as lipid few appear to exceed 40%. From the survey these species are *Botryococcus braunii* (53% lipid), *Dunaliella salina* (47%), and *Radiosphaera negevensis* (43%). *Scenedesmus obliquus* (-49%), *Nannochloris* sp. (49%) *Ourococcus* sp. (50%), *Scenedesmus obliquus* (-49%), *Nitzschia palea* (-40%) *Navicula pelliculosa* (-45%).

Monalanthus salina (-70%) and *Biddulphia qurita* (-40%), as well as *Botryococcus braunii* (-70%) as good lipid producers. *Chlorella pyrenoidosa*, at about 37% lipid, would also seem to be potentially useful.

Botryococcus braunii has an established reputation as a lipid producer having been implicated in the formation of oily droplets of the type known as Boghead Coal where it was found to contain up to 86% of its mass as hydrocarbon. This would appear to be an exceptionally high figure and 30% would appear more reasonable under laboratory conditions, the organism can be grown very slowly with doubling times of up to 6 weeks. Nevertheless, the organism can be grown in laboratory conditions to produce over 50% of its biomass as lipid with this lipid containing 20% hydrocarbon. Hillen shown that hydrocracking of the hydrocarbons, whose principal component is botryococcene (Fig. 26.2), gives 67% gasoline.

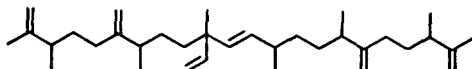


Figure 26.2 : Structure of botryococcene, a major lipid component from *Botryococcus braunii* (from Hillen et al., 1982

The future exploitation of this alga would therefore appear to lie as an alternative means of producing fuel oil. An added advantage with this species is that, although classed as a freshwater alga, it can be cultured on media with osmotic potentials up to those of seawater with no adverse effects.

Of the more conventional algae, species of *Chlorella* (either *C. pyrenoidosa* or *C. vulgaris*) would appear to be worth investigation further. Shifrin (1984) has shown that a copper-tolerant clone of *C. vulgaris* produces 40% lipid content in about 9 days culture whereas the copper-sensitive strain produces less than 30% lipid. A recent report from the Solar Energy Research Institute, California has also indicated *Chlorella* to be an organism of choice for the production of oils to act as vegetable oil substitutes.

With the possibilities of genetic improvement or of strain selection and improvement, perhaps by utilizing copper tolerance as indicative of a high lipid content as in Shifrin's work, the way seems to be open for striking developments in this field. The opportunities for large scale algal cultivation in such countries as

Israel would appear to offer excellent possibilities of deriving acceptable replacements for existing plant seed oils.

The fatty acids produced by potentially useful algae are given in Table 26.5. As will be seen, algal lipids are marked by their exceptionally high proportions of polyunsaturated fatty acids. Unfortunately as these are the type which are also found in fish oil, the frequent complaint against algal lipids is their unpleasant 'fishy' odour. The desirability of including polyunsaturated acids in the diet might suggest that a proportion of algal oils could be mixed with a more saturated or monounsaturated plant oil, viz. palm oil or rapeseed oil (see Table 26.2) to give a nutritionally acceptable blend.

Yeasts and Moulds

Organisms

The number of oleaginous organisms is not extensive. For the yeasts, the list is given in the Table 26.6 some 16 classified species have been reported as producing better than 25% lipid. In addition there are two species identified only to the genus level (*Candida* sp. 107 and *Lipomyces* sp. 33). It should be pointed out that many of the designated species contain numerous strains, some of which readily attain high lipid contents whereas others bearing the same name do not.

New oleaginous species are continually being added to the list. It is possible that other known yeasts could prove to be oleaginous if they were grown under the appropriate conditions. The prerequisite for lipid accumulation is the possession of ATP:citrate lyase and it is this activity which should be determined in order to ascertain if a given yeast might be capable of lipid accumulation. The enzyme has been found in all oleaginous yeasts so far examined (Boulton and Ratledge, 1981). It is not present in non-oleaginous species, nor even in nonoleaginous strains of organisms such as *Lipomyces starkeyi* and *L. lipofer*. It is therefore an extremely powerful determinant for lipid production.

The number of oleaginous moulds is considerably greater than yeasts. However, to give a list for moulds similar to that given in Table 26.6 for yeasts could be misleading as much of the information on oil production has come from work in which the moulds have

Table 26.5 : Fatty Acid Composition of Selected Oleaginous Algae

Alga	Relative (%) (w/w)												
	14:0	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	18:3 (□)	18:3 (□)	18:4	20:U
<i>Spirulina platensis</i>	1	43	3	5	—	—	1	6	22	0.3	18	—	—
<i>Chlorella pyrenoidosa</i>	1	21-34	2-8	8-16	3-13	—	0.3-3	4-17	19-30	8-26	—	—	—
<i>Chlorella vulgaris</i>	2	26	8	7	2	—	2	2	34	20	—	—	—
<i>Scenedesmus actus</i>	1	15	1	tr	4	20	1	8	20	30	0.4	—	—
<i>Dunaliella primolecta</i>	5	11	10	8	7	6	tr	6	6	10	2	7	14
<i>Navicula pelliculosa</i>	3	9	31	3	18	—	—	6	4	2	—	—	19
<i>Nizschia palea</i>	6	23	45	4	2	—	—	3	—	—	—	—	18

tr = trace.

been grown as 'felts' in static culture, often for two to three weeks. In the authors' experience, many of the claims from early work cannot be repeated when the moulds are regrown in a stirred tank reactor. A list of some oleaginous moulds is given in Table 26.7 but the veracity of this cannot be verified. Whether the presence of ATP: citrate lyase could be used to indicate lipid-accumulating ability, as with yeasts, is completely unknown as no systematic survey has so far been carried out with moulds to examine this property.

Table 26.6 : Oleaginous Yeasts

Yeasts	Lipid (%)
<i>Candida curvata</i> (spp. R and D)	51-58
<i>Candida guilliermondii</i>	25
<i>Candida</i> (<i>Saccharomycopsis</i> , now <i>Yarrowia</i>) <i>lipolytica</i>	36
<i>Candida paradipolytica</i>	32
<i>Candida</i> sp. 107 (NCYC 911; CBS 329.80)	42
<i>Cryptococcus terricolus</i> (syn. <i>C. albidus</i>)	55-65
<i>Endomyces vernalis</i> <i>Endomycopsis vernalis</i> } (= <i>Trichosporon pullans</i> (q.v.)	
<i>Hansenula saturnus</i>	28
<i>Lipomyces lipofer</i> (= <i>lipofera</i> , <i>lipoferus</i>)	64
<i>Lipomyces starkeyi</i>	63
<i>Lipomyces tetrasporus</i> (sp. 5011F)	64
<i>Lipomyces</i> sp. no. 33 (= <i>L. lipofer</i> ?)	67
<i>Rhodospiridium toruloides</i>	66
<i>Rhodotorula glutinis</i> (syn. <i>R. gracilis</i> and <i>R. suganii</i>)	74
<i>Rhodotorula graminis</i>	41
<i>Rhodotorula mucilaginosa</i>	28
<i>Trichosporon cutaneum</i>	45
<i>Trichosporon pullulans</i> (syn. <i>Geotrichum candidum</i>)	65
<i>Trigonopsis variabilis</i>	40

Triacylglycerols and fatty acids

The major accumulating lipid of yeasts and fungi is the triacylglycerol fraction which can account for up to 92% of the total

lipid of a cell. Where positional analysis of the fatty acyl residues has been carried out, the *sn*-2 position of the glycerol appears to be almost entirely occupied by unsaturated fatty acids. This, therefore, is the same type of distribution which occurs in plant seed oils but differs from that in animal fat where saturated fatty acids occur on the *sn*-2 position.

In their detailed analysis of the oil from *Cunninghamella blakesleeana*, Jack distinguished between the *sn*-1 and *sn*-3 positions of the glycerol. These two positions were not acylated by the same fatty acids: the *sn*-1 position contained 65% of its acyl groups as palmitic acid (16:0) and oleic acid (18:1) whereas the *sn*-3 position contained only 39% palmitate and 10% oleic acid, the remainder being accounted for by linoleic (18:2) and linolenic (18:3) acids.

The fatty acids of yeasts are usually in approximate order of abundance: oleic, palmitic, linoleic and stearic acids. Linolenic acid (18:3) and palmitoleic acid (16:1) may be found a few per cent in some cases. A list of the fatty acids of the major oleaginous species is given in Table 26.8. It should be appreciated that these values are only a guide as

Table 26.7 : Some Moulds which have been claimed to be Oleaginous

<i>Mould</i>	<i>Lipid (%)</i>
Entomophthorales	
Entomophthora conica	38
Entomophthora coronata	47
Entomophthora obscura	34
Entomophthora thaxteriana	26
Mucorales	
Absidia corymbifera	27
Absidia spinosa	28
Blakeslea trispora	37
Cunninghamella elegans	44
Cunninghamella homothallica	38
Mortierella isabellia	63
Mortierella vinacea	66
Mucor circinelloides	65
Mucor ramannianus	56
Mucor spinosus	47
Rhizopus arrhizus	49
Rhizopus oryzae	32
Ascomycetes	
Aspergillus fischeri	53
Aspergillus nidulans	25
Aspergillus ochraceus	48
Aspergillus terreus	57
Fusarium sp. N-11	39
Fusarium oxysporum	51
Gibberella fujikuroi	48
Penicillium lilacinum	51
Penicillium spinulosum	64
Hyphomycetes	
Cladosporium herbarum	49
Pellicularia	
Pellicularia praticola	39
Basidiomycete	
Ustilago zeae	59

variations, sometimes considerable, can be achieved by cultivating the organism of choice under different conditions. Such conditions would include variation in oxygen tension, choice of growth substrate, nitrogen source, growth temperature as well as the growth rate of the organism itself.

Table 26.8 : Fatty Acyl Groups of Lipids from Various Oleaginous Yeasts

Yeast*	Relative%						
	14:0	16:0	16:1	18:0	18:1	18:2	18:3
<i>Candida curvata</i> D	tr	36	-	14	40	6	-
<i>Candida guilliermondii</i>	-	21	6	5	61	5	1
<i>Candida</i> 107	3	44	5	8	31	9	1
<i>Hansenula saturnus</i>	1	24	3	4	30	25	12
<i>Lipomyces lipofer</i>	2	16	3	3	62	9	1
<i>Lipomyces starkeyi</i>	-	40	6	5	44	4	-
<i>Rhodotorula glutinis</i>	2	16	-	7	39	24	1
<i>Rhodotorula gracilis</i>	-	25	-	12	51	11	1
<i>Trichosporon Cutaneum</i>	-	30	-	13	46	11	-

* All yeasts were grown on glucose except *T. cutaneum* which was grown on lactose.

The fatty acids of moulds show a greater range and diversity than yeasts. Members of the Entomophthoraceae are characterized by the presence, often in substantial amounts, of shorter chain fatty acids (C_{10} to C_{14}) as well as still containing C_{18} polyunsaturated acids. Branched chain acids have also been reported in these organisms. The fatty acids of three oleaginous species are given in Table 26.9. Fatty acids of other fungi are given in Table 26.10. As with yeasts, considerable variations in the fatty acyl composition may occur under different growth conditions or with different substrates and nutrients being used.

Hydroxy fatty acids, including ricinoleic acid (12-hydroxystearic acid), occur up to 62% of the total lipid in *Claviceps* spp. Their synthesis, though, only occurs in the sclerotial form of the mould which is a form difficult to propagate in a stable manner in the laboratory. Although *Claviceps* lipid may be thought of as an attractive alternative to castor oil, the main value of any large-scale cultivation of this mould would be that it also produces ergot alkaloids when in its sclerotial form. The commercial value of the culture would then be in the high value minor component rather than in its oil. Although it would not seem impossible to extract the

Table 26.9 : Relative % of Fatty Acids in Lipids from three Oleaginous Species of Entomophthora Grown on Glucose in Shake Culture for 120 h (from Popova *et al.*, 1980)

	10:0	12:0	13:0	14:0	16:0	16:1	16:2	18:0	18:1 ^a	18:2 ^a	18:3 ^a	19:0	20:4
<i>E. conica</i>	–	1.7	0.6	11.7	18.0	28.0	1.0	2.9	20.1	2.0	1.4	–	8.8
<i>E. coronata</i>	2.2	17.9	2.7	29.5	12.9	0.6	–	1.9	12.3	9.5	tr	5.2	2.1
<i>E. thaxteriana</i>	1.8	0.4	0.5	8.7	25.5	17.3	0.6	4.6	19.7	3.8	1.5	–	7.0

All samples also contained traces of 15:0, 17:0, 20:1, 20:2 and 20:3; 11:0, 17:1 and 17:2 were also present in *E. coronata*, with traces of 22:0 and 24:0 in *E. conica*

* Mixture of positional isomers: for 18:1, two isomers; 18:2, three isomers; 18:3m two isomers.

Table 26.10 : Fatty Acids of the Lipid from Various Oleaginous Moulds

	16:0	16:1	18:0	18:1	18:2	18:3	20+
<i>Absidia corymbifera</i>	23	1	20		22	21	3 ^a
<i>Blakeslea trispora</i>	18	4	2	10	62	11a	3
<i>Cunninghamella elegans</i>	20	2	5	40	19		–
<i>Mortierella isabellia</i>	35	6	3	42	8	5a	–
<i>Rhizopus oryzae</i>	20	2	8	48	11	6a	–
<i>Aspergillus nidulans</i>	20	3	12	39	13	9a	–
<i>Aspergillus terreus</i>	23	0.1	0.3	14	39	21	–
<i>Cladosporium herbarum</i>	31	tr	13	35	18	0.6	–
<i>Fusarium N₁</i>	21	1	–	76 ^a			–
<i>Fusarium oxysporum</i>	17	0.4	8	20	47	10	–
<i>Pellicularia praticola</i>	12	0.3	7	20	47	10	–
<i>Penicillium lilacinum</i>	28	0.3	13	37	20	0.1	–
<i>Penicillium spinulosum</i>	15	1	7	42	31	1	–

In all species there are traces of 12:0 and 14:0 fatty acids

^a 6.9.2-18:3 (γ-linolenic acid); in all other cases this is the 9.12.15-18:3 isomer (α-linolenic acid). ^b Said by authors to be 20:0 but probably confused with α-linolenic acid, ^c18:0 18:1 and 18:2 not distinguished.

ricinoleic acid from such cultures, and thus produce a valuable by-product, the volume likely to be obtained would be so insignificant (in terms of the quantities produced by the castor oil industry) that its recovery would probably not be worthwhile.

Steroids

Sterols can be produced in some abundance by eukaryotic microorganisms and have been obtained in commercial quantities by extraction of spent fungal mycelium recovered from various fermentation processes. As far as we are aware, few of these processes are still carried out though ergosterol is produced in one or two instances by extraction of *Saccharomyces cerevisiae*, though it is not certain whether the organism is specifically grown for this purpose or whether extraction is only carried out on spent brewers' yeast.

Ergosterol (see Fig. 26.3) is a common constituent of most yeasts and moulds. In a detailed study of 558 yeast cultures, including 240

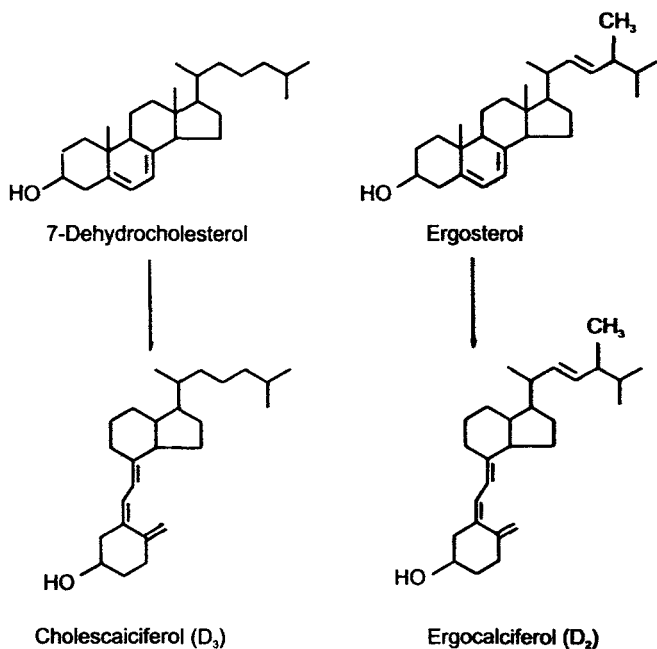


Fig. 26.3 : Relationship of ergosterol and 7-dehydrocholesterol to vitamins D₂ and D₃

species of *S. cerevisiae*, Dulaney found that species of *Saccharomyces* were the only ones which consistently produced more than 0.1% of their biomass as ergosterol. They identified eight cultures of *S. cerevisiae* which produced ergosterol from 7 to 10% of the dry cell biomass. The best of these yeasts could be grown to produce cell dry weights in excess of 30 g l⁻¹ giving an ergosterol yield of between 3 and 4g per litre of culture. Some commercial production of ergosterol appears to have been carried out using the same or similar high yielding strains. Owing to industrial secrecy, it is not known whether this production is still ried out today.

The main application of ergosterol is its use as an analogue for cholecalciferol, vitamin D₃-Ergocalciferol-vitamin D₂-(see Fig. 26.3) is formed from ergosterol by the action of ultra violet light in the same way as vitamin D₃ is formed from 7-dehydrocholesterol. However it has a decreased effectiveness in some animals; in chickens, for example, it is only about as 10% as good. The real need therefore is for 7-dehydrocholesterol rather than ergosterol but an inexpensive source of this pro-vitamin D₃ material is not readily available. Recent work would seem to indicate that this problem may be resolved by using yeast mutants. As yet there is still some way to go before this goal can be realized though other potentially valuable sterols have been identified.

Ergosterol is also a common constituent in most moulds. It has been reported as accounting for 92% of the total sterols in *Aspergillus fumigatus* which can produce put up 5% of its cell dry weight as sterols. As stated at the beginning of this section, the natural occurrence of relatively high levels (at 2 to 3% of the biomass) of sterols in various moulds has led to some extractions being carried out on mycelium recovered from antibiotic and other fermentation processes. However, the problems of coextraction of residual antibiotics, or of there being potentially deleterious materials, would now necessitate adequate toxicological tests being carried out before the fungal sterols may be permitted back into the food chain.

Other lipids

A wide variety of lipids, besides triacylglycerols and fatty acids, can be obtained from eukaryotic microorganisms. Of these, probably the greatest commercial attention has been paid to the carotenoids, of which a wide variety of molecular species are produced. The

principal carotenoid of interest is β -carotene; not only is it an important red colorant but it also acts as a pro-vitamin A compound.

Phospholipids occur in all living systems. They may reach up to about 5% of the cell biomass in bacteria, especially in those grown on methane or methanol where extensive phospholipid membranes occur as part of the mechanism for the organism being able to deal with these C_1 compounds. Phospholipids can provide useful chemical properties with the combined presence of a large polar group such as choline, $-OCH_2CH_2N^+(CH_3)_3$, attached to a large non-polar group – the diacylglycerol moiety. Such materials are potential emulsificants and surfactants. The extracted lipid has been given the name Biolipid and is described as a dark brown, slightly viscous, combustible liquid with a characteristic odour. It has a high content of alkanes (45 to 55%), which reinforces the view of the unacceptable nature of the original whole yeast, contains phospholipids from 20% to 30%, some triacylglycerols (10-20%), free fatty acids (up to 10%) with small amounts (1%) of sterol and ubiquinone. Thus, apart from the inert residual hydrocarbons, which will be of the gas oil or kerosene variety, the major component of this material is the phospholipid which then gives the lipid useful boundary surface properties. A variety of technical applications for the whole oil have been suggested: an additive to fuel oil to improve dispersion and flame temperature; a mould-releasing agent in the casting of concrete parts in the building industry; and a plant protective agent; an additive to bitumen to improve its adhesion characteristics; and a protective layer or conditioning layer over deliquescent chemicals such as granular fertilizers and potash salts. The individual components have been separated and some applications for each have been suggested.

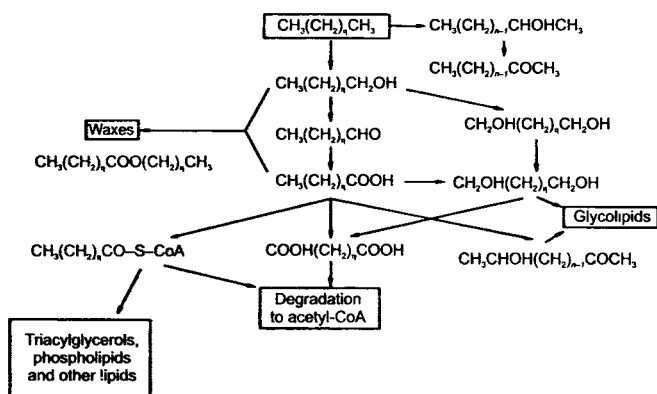
Substrates

Hydrocarbons

The use of hydrocarbons as a means of producing yeast either for food or for fodder has fallen into disfavour, though perhaps only temporarily. Thus if the production of lipids were to be contemplated from hydrocarbons, they would have to be considered for technical uses only.

Hydrocarbons, and alkanes in particular, have the advantage over other substrates in that they can predetermine the chain length of the ensuing fatty acids found in the extracted lipids. This may be of considerable advantage if a lipid with particular fatty acid substituents should be wanted for any reason. Hydrocarbons, in general, also lead to the greater production of lipid, as a percentage of the cell biomass, than do carbohydrate. This may again be of advantage where a product such as a wax may be wanted but is normally only found as a small percentage of the total biomass.

Besides being useful for the production of specific fatty acids which are then recoverable as triacylglycerols or phospholipids, hydrocarbons can lead to the production of both ω and $\omega-1$ hydroxy fatty acids and dicarboxylic acids. These arise by microbial attack of the alkane chain occurring at both ends of the molecule. The hydroxy fatty acids which are formed with *Torulopsis bombicola* and related species are recovered as esterified to a disaccharide sophorose. These sophorosides can be produced in some quantity and may be thought of as a biological Tween. Various applications of these compounds have been considered but none has seemingly warranted commercial production; the cheapness of producing similar molecules by chemical means, e.g. fatty acyl sucroses, has overridden all possibility of being able to produce such materials competitively by microbial means.



$n = 8$ to 16 , especially 11 to 14

Fig. 26.4 Pathways of alkane oxidation and assimilation in microorganisms

ω and $\omega-1$ Hydroxy fatty acids, isolated from *T. bon.bicola* have been used to produce polyester material of high molecular weight



where, if $\text{R}=\text{CH}_3$, $n=15$; if $\text{R}=\text{H}$, $n=16$. However the intrinsic properties of the polyester were apparently not substantially different from those of synthetic polyesters to warrant further consideration as a commercially viable product.

Dioic acid production from alkanes has been well documented. The route of biosynthesis is usually considered to be via the ω -hydroxy fatty acid as given in Figure 26.4 have indicated the dihydroxy acid may be an intermediate.

Fatty Acids, Soapstocks and Oils

Hydrocarbons have severe limitations when it comes to producing lipids intended for incorporation into human foodstuffs. Embargoes, arising either indirectly by public opinion or by government legislation, on the use of alkanes as fermentation feedstocks do not however apply to fatty acids which can metabolically produce the same alterations in lipid compositions as alkanes. Various patents have been taken out which have sought to demonstrate that desirable lipids can be produced by cultivating appropriate yeasts, usually of the genus *Candida*, *Torulopsis* and *Trichosporon*, though *Saccharomyces cerevisiae* and *C. utilis* have also been used, on a mixed carbon source which includes a fatty acid or material containing fatty acid. The fatty acids or oils may be up to 20 g l⁻¹ in the growth medium and, like alkanes, these then lead to high lipid contents: up to 65% and 67% have been reported. Equally importantly, high relative percentages of stearic acid (if stearic acid had been reported. Equally importantly, high relative percentages of steric acid (if stearic acid had been included in the medium) may also be achieved in the yeast oil. This, it is claimed, can then lead to a yeast lipid which has some of the characteristics of cocoa butter.

Oils themselves can be presented to yeasts, again with the object of producing triacylglycerols with altered acyl substituents. The possibilities of upgrading the cheap vegetable oils, such as palm oil, to more expensive materials have been considered. Such a process relies upon the various lipases of the organism carrying out the initial hydrolysis; the ensuing fatty acids are then incorporated directly into new triacylglycerols in the same manner

as occurs when alkanes are used as substrate. Lipases though may be isolated from microorganisms and, as immobilized preparations, then used to carry out transesterification reactions either between two different oils between an oil and a fatty acid. Such processes are under active consideration by several companies to produce high value-added commodities such as cocoa butter.

Other Substrates

A wide variety of substrates have from time to time been considered for the production of oils and fats. These include various starchy crops and wastes, molasses, whey, peat hydrolysates and ethanol. The only substrate which is abundant and is not too expensive but which cannot be used by oleaginous microorganisms is methanol. Although methanol-utilizing yeasts are well known, none has been described which accumulates more than 15% lipid, though even here the triacylglycerols account for 72% of the total lipid.

Future Prospects

The future prospects for microbial oils might be seen to lie in three possible areas: (1) as substitutes for high value plant oils; (2) as novel materials unavailable from other sources (3) as a saleable end product from waste processing.

With the first prospect, the higher the value of the oil to be replaced, the smaller tends to be its market. The highest priced oils have only small markets, perhaps of the order of only a few tonnes per annum. The production of small tonnages of microbial oils is of course much more expensive than production in bulk and it requires a perceptive understanding of the market to identify a potentially useful target. With respect to oils which may be produced on a much larger scale, it has to be said that few, if any, of the major oils and fats companies have much insight or commitment into fermentation technology. They therefore usually remain sceptical of the concept of a microbial oil being a satisfactory alternative to a plant oil. Moreover they are extremely reluctant to enter an entirely new field of high technology activity and begin production of microbial oils for themselves. Thus, if microbial oils were to be produced they would have to be produced by a company which was not in the oils and fats industry. Not unnaturally, fermentation companies are reluctant to produce

materials for another highly competitive industry. They will certainly not undertake production of oils for marketing themselves as this is an area of commercial activity in which they have no expertise but which they know to be very traditional in outlook.

Microbial lipids which are novel materials in their own right do exist but materials similar to them can often be produced chemically, and this usually means cheaper.

Single cell protein (SCP) has long been produced as a valuable adjunct to waste processing. The SCP, though, is only suitable for animal feed and as such commands little more than the price of soybean meal. It has been argued elsewhere (Rateldge, 1982) that SCO (single cell oil) could be a more valuable product than SCP as the oil; at worst, it would compete with the lowest priced oils which are already some 3 to 3.5 times higher in value than animal feed protein. Credit would also be obtained for the defatted microbial biomass which would, like its oilseed meal counterpart, be sold as animal fodder. The argument that SCP production is uneconomical no matter what substrate is used may be true when the substrate has to be purchased but when SCP is a saleable end-product arising from waste which would otherwise be costly to dispose of, then the argument no longer holds. Single cell oil has the advantage over SCP in that it can be used for technical purposes and therefore expensive toxicological trials are unnecessary. If the waste in question is such that the end-product could not be returned into the food chain, then clearly SCP is not a sensible product, but SCO could be.

There are few materials which a range of microorganisms can each produce and which can find a range of technological applications. With fermentation products such as ethanol or butanol one is restricted to using a small number of organisms. Moreover the substrates which need to be degraded in a particular waste-processing system may not be suitable for these particular organisms to convert into ethanol or butanol. Methane production is the resort of the unimaginative. With the range of oleaginous organisms which are now available, it would be surprising if one could not be found which could usefully attack almost any waste organic material. Microbial oils therefore be an important addition to the biotechnologist's armoury.

Microbial Polysaccharides

Exopolysaccharides produced by a wide variety of microorganisms are water soluble gums which have novel and unique physical properties. Because of their wide diversity in structure and physical properties microbial exopolysaccharides have found a wide range of applications in the food, pharmaceutical and other industries. Some of these applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents. These biopolymers are rapidly emerging as a new and industrially important source of polymeric materials which are gradually becoming economically competitive with natural gums produced from marine algae and other plants. The potential use of genetically modified microorganisms under controlled fermentation conditions may result in the production of new exopolysaccharides having novel superior properties which will open up new areas of industrial applications and thus increase their demand.

Sources and Applications of Exopolysaccharides

Microbial polysaccharides, which serve different functions in a microbial cell, may be distinguished into three main types: (a) intracellular polysaccharides which may provide mechanism for storing carbon or energy for the cell; (b) structural polysaccharides which are components of cell structures such as lipopolysaccharides and teichoic acids present as integral components of cell wall; and (c) extra cellular polysaccharides referred to in this chapter as exopolysaccharides.

Sources of Microbial Exopolysaccharides

Production of exopolysaccharides is found in many species of Gram-positive and Gram-negative bacteria, some algae and many fungi. Table 27.1 summarizes the production of exopolysaccharides

by various microorganisms using a wide variety of substrates, such as glucose, fructose, sucrose, lactose, hydrolyzed starch, methanol and different hydrocarbons.

Table 27.1 : Production of Exopolysaccharides by Various Microorganisms

<i>Product</i>	<i>Substrate</i>	<i>Microorganism</i>	<i>Yield</i>
Alginate	Sucrose	<i>Azotobacter vinelandii</i> NCIB 9068	5% batch 25% batch 45% continuous
Polymer of: D-glucose, D-mannose, D-ribose, 6-deoxy- L-mannose	2% Glucose	<i>Xanthomonas fuscans</i>	35%
Curdian succinoglucon	Glucose	<i>Alcaligenes faecalis</i> var. <i>myxogenes</i> 10C3	
Curdian types	5% Glucose	<i>Alcaligenes faecalis</i> var. <i>myxogenes</i> 10C3 LFO 13140	50%
Erwina gum (Zanflo)	Lactose, hydrolyzed starch	<i>Erwina tahitica</i>	n.a.
Polymer of : D-glucose 81.9%, L-rhamnose 14%, L-glucose 0.7%, D-mannose 1.9%, D-galactose 1.5%	1% w/v methanol	<i>Methylocyctis parvus</i> OBPP	62% fed
Polysaccharide	4.55% methanol	<i>Methylomonas mucose</i> NRRLB-5696	45.2%
Polysaccharide	0.3% v/v methanol	<i>Methylomonas methan-</i> <i>olica</i> M13V mutant	
Polymer of : galactose, glucose, mannose, glucuronic acid	1% methanol	<i>Pseudomonas</i> sp. <i>viscogena</i> TS-1004	21%
Polymer of : glucose, galactose, rhamnose, mannose, acetatepyruvate	2% glucose	<i>Pseudomonas</i> NCIB 11264	n.a.

contd....

Table 27.1 – contd...

Product	Substrate	Microorganism	Yield
PS-60 gum: glucose 41%, rhamnose 30%, uronic acid 29%		<i>Pseudomonas</i> sp.	n.a.
Levan	2% sucrose	<i>Zymomonas mobilis</i> NCIB 8938	<2%
Seleroglucan	3% glucose	<i>Selerotium rolfsii</i> ATCC 15206	1.5-2.2% w/v
Seleroglucan	5% w/v starch	<i>Selerotium delphinii</i> , <i>S. glucanicum</i>	1.4% w/v 1.8% w/v
PS-7 gum: glucose 73%, rhamnose 16%, glucuronic acid 11%	3% glucose	<i>Beijerinckia indica</i> var. <i>mysogenes</i>	n.a.
Pullulan	5% sucrose	<i>Aureobasidium</i> <i>pullulans</i> S-1	50-60%
Phosphomannan	Hydrolyzed whey, 4.4% sugars	<i>Hansenula holstii</i> NRRL y-2448	20.0%
Levan	6% lactose	<i>Alcaligenes viscosus</i> NRRL B-182	2.5%
Xanthan gum	6% lactose	<i>Xanthomonas</i> <i>campestris</i> NRRL B-1459	38.3%
Galactoglucan	6% lactose	<i>Zoogloa ramigera</i> NRRL B-3669	55.6%
Polymer of : galactose, glucose, glucuronic acid	6% lactose	<i>Arthrobacter viscosus</i> MRRL B-1973	0.7%
O-acetylated acid polysaccharide: glucose, mannose, mixture galactose	10% v/v C ₁₂ -C ₁₇ <i>n</i> -paraffin	<i>Corynebacterium</i> sp. <i>Brevibacterium</i> sp.	n.a.
Gellan gum (S-60 polysaccharide)	Carbohydrate	<i>Pseudomonas elodea</i> ATCC 31461	

Applications of Microbial Exopolysaccharides

Microbial exopolysaccharides is found a wide range of applications in the chemical, food and pharmaceutical industries. In view of their unique and novel chemical and physical properties,

microbial exopolysaccharides are being used as gelling agents, emulsifiers, stabilizers, binders, coagulants, lubricants, film formers, thickening and suspending agents. Typical applications of an exopolysaccharide, xanthan gum, are summarized in Table 27.2.

Table 27.2 : Major Food and Industrial Applications of Xanthan Gum

Food Applications

Dressings (high oil, low oil, no oil);

Relishes and sauces; Syrups and toppings;

Starch based products (canned desserts, sauces, fillings, retort pouches);

Dry mix products (desserts, gravies, beverages, sauces, dressings);

Farinaceous foods (cakes);

Beverages; Dairy products (ice cream, shakes, processed cheese spread, cottage cheese); Confectionery.

Industrial Chemical Applications

Flowable pesticides; Liquid feed supplements;

Cleaners, abrasives and polishes;

Metal working; Ceramics, Foundry coatings; Texturized coatings;

Slurry explosives; Dye and pigment suspensions.

Oil Field Applications

Drilling fluids (muds) : Workover and completion fluids; Enhanced oil recovery (polymer flooding).

Structure and Composition of Exopolysaccharides

Exopolysaccharides produced by a wide variety of microorganisms are important biopolymers which have unique physical and chemical properties. Their diversity in structure and composition makes these biopolymers very versatile in a wide range of industrial applications. Of particular importance is the rheological characteristics of exopolysaccharides, which are influenced by their structure and composition. The structure and composition of microbial exopolysaccharides depends on many different factors, such as microbial species, nature of substrate and other fermentation conditions. In this section we describe the structure and composition of a few selected microbial exopolysaccharides as an example to illustrate the nature and diversity of these biopolymers.

A Brief Classification of Polysaccharides

Microbial polysaccharides result from the condensation of monosaccharide units by eliminating water between the C₁ hydroxyl group of one unit and an available hydroxyl group of another monosaccharide. Polysaccharides may be classified into two types namely, homopolysaccharides, which contain only one type of sugar moiety, and heteropolysaccharides, which contain two or more sugar moieties. Heteropolysaccharides are usually produced by microorganisms from any utilizable carbon source and complex enzyme systems are usually involved, whereas homopolysaccharide synthesis involves a single or simple enzyme system. Examples of homopolysaccharides include pullulan and dextrans; xanthan gum is a typical example of a heteropolysaccharide. Depending on the structural arrangement of the monomeric units, polysaccharides are either linear or branched. Another important distinction of polysaccharides is based on their charge properties and they may be classified as naturally anionic, neutral or cationic. Some anionic microbial exopolysaccharides include the following: xanthan gum, phosphomannan and alginate. Neutral exopolysaccharide examples include: levan, scleroglucan, pullulan, dextran and curdlan. Some polysaccharides have anionic properties and they contain acidic groups, such as carboxyl, phosphate or sulfate. Other polysaccharides in their natural state may have some free amino groups and as a result these biopolymers possess cationic properties.

Xanthan Gum

Xanthan gum produced by *Xanthomonas campestris* is a branched anionic heteropolysaccharide. As shown in Figure 27.1, the xanthan gum biopolymer has a repeat unit of five sugars and pending on the microbial source and fermentation conditions the molecular weight may be greater than 10⁶. Xanthan contain D-glucose, D-mannose, D-glucuronate and variable amounts of acetate and pyruvate. The macromolecule has a cellulose backbone (β -(1 \rightarrow 4) linked glucose) with a trisaccharide sidechain composed of two mannose and one glucuronate residue (Figure 27.1). The acetate and pyruvate substituents are attached to the mannose residues (Figure 27.1). In addition to the different sugar units acetyl and pyruvate acetal groups, xanthan gum also contains monovalent cations as shown in Figure 27.1. There is some debate as a result of

X-ray diffraction studies where xanthan gum exists as a single or double helix. Xanthan gum in aqueous solutions has many interesting properties resulting in the use of this biopolymer in a wide variety of industrial applications (see Table 27.2). The viscosity of aqueous solutions of xanthan gum is almost entirely independent of temperature over a range 10-70 °C, and is also fairly constant for pH between 6 and 9. Xanthan gum aqueous solutions have very good compatibility with high concentrations of salts and exposure to temperatures up to 80 °C for a long period of time has little effect on physical properties.

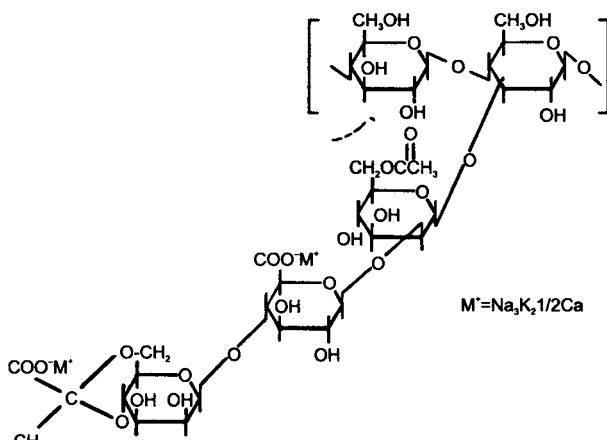


Fig. 27.1 : Polymeric structure of xanthan gum.

Dextran is primarily produced commercially by two microorganisms *Leuconostoc mesenteroides* and *Leuconostoc dextranicum*. Depending on the microbial species employed, the type of substrate and fermentation conditions, a wide variety of dextrans may be produced with structures which are slightly or highly branched. Dextran is also synthesized from sucrose using cell-free culture filtrates of *Leuconostoc mesenteroides* strain. Dextran is a branched neutral homopolysaccharide which is composed exclusively of α -D-glucopyranosidic residues, and 95% of these residues are linked through carbons 1 or 1 and 6. The remaining 5% of the α -D-glucopyranosidic residues in the macromolecule carry branches of about one or two glucose units attached at carbon 3

positions. Figure 27.2 shows the structure of dextran. One of the most successful applications of dextrans is in the manufacture of a series of important molecular sieves. The degree of cross-linking of dextran with different epoxy compounds and sodium hydroxide, determines the pore size and water regain value of these molecular sieves and thus their molecular exclusion characteristics.

Alginate

Although most of the commercial alginate produced today is derived primarily from the sea kelp *Macrocystis pyrifera*, microbially derived alginates are under development and their future is very promising. The wide variety of products obtained from *Azotobacter vinelandii* have been found to possess physical properties similar to the alginates derived from marine algae. The biopolymers derived from *azotobacter vinelandii* have a wide range of molecular weights and it has been postulated that the extracellular enzyme alginate lyase may play an important role in the molecular weight of alginates. Alginate is a general term used to describe the salts of alginic acid, the most notable of which is sodium alginate. Alginic acid is a weak organic acid which readily forms salts with different bases. Alginic acids are linear polysaccharides composed of varying proportions of β -(1 \rightarrow 4) linked D-mannopyranosyluronic acid and α -(1 \rightarrow 4) linked L-guluronic residues in block and alternating sequences in the linear chain. The presence of L-guluronic acid in the alginic acid macromolecule is important because increasing its content improves the gelling characteristics of alginate in the presence of calcium ions. Sodium alginate is used widely in research as a gelling agent to immobilise a wide variety of cells, such as microbial cells, plant and mammalian cells.

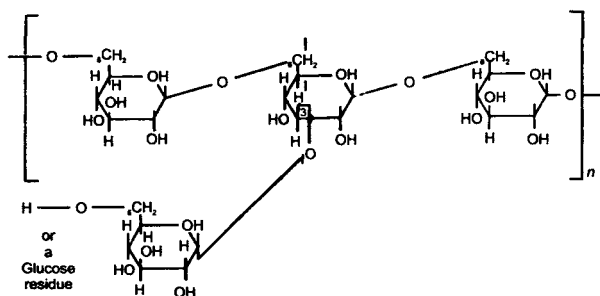


Fig. 27.2 : Structure of dextran exopolysaccharide.

Alginates have ion-exchange properties similar to ion-exchange resins. The relative affinities of divalent metal ions depend on the relative amounts of D-mannuronic and L-guluronic acid units which are present in the macromolecule.

Pullulan

Pullulan is a neutral linear homopolysaccharide which is composed of glucose units polymerized into repeating maltotriose units (Figure 27.3). The repeating maltotriose units are linked by α -(1 \rightarrow 6) glucosidic bonds, and within each maltotriose unit the glucopyranose units are linked by α -(1 \rightarrow 4) glucosidic bonds. Because its structure pullulan has been used as a source of maltotriose, and also as a substrate to measure the activity of the enzyme α -(1 \rightarrow 6) glucanohydrolase (pullulanase), an important enzyme used to depolymerize amylopectin. The unique structure and physical properties of pullulan form the basis for a wide range of industrial applications such as adhesives, fibers, molded articles, coatings, films and esterified derivative applications. Starch hydrolysates, at a concentration of 10%, were used to produce pullulan with high yields of 75%.

Scleroglucan

Scleroglucan is a highly branched neutral homopolysaccharide composed of glucose units. This exopolysaccharide is produced from different carbohydrate substrates using *Sclerotium glaucum* and other related *Sclerotium* fungal species. The scleroglucan macromolecule is highly branched and has a backbone chain of

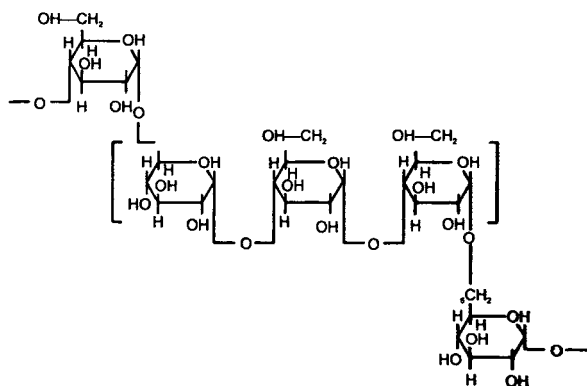


Fig. 27.3 : Structure of pullulan exopolysaccharide.

about 90 β -(1 \rightarrow 3) linked glucopyranosyl residues and every third or fourth backbone residue is linked to a single D-glucopyranosyl side group by β -(1 \rightarrow 6) glucosidic bond.

Microbial Biosynthesis of Exopolysaccharides

A systematic perusal of the literature revealed that, despite the commercial importance of exopolysaccharide producing microorganisms, very little is known about the biochemical pathways involved during the biosynthesis of these different exopolysaccharides. A good understanding of the pathways involved in exopolysaccharide biosynthesis is important because this information can be used to control and optimize rates and yields of fermentation, as well as the physicochemical characteristics of exopolysaccharides.

The majority of microbial exopolysaccharides are assumed to be synthesized within the cell in an analogous mechanism to that involved in cell wall synthesis. Very few exopolysaccharides have been reported to be synthesized outside the cell.

Cell Biosynthesis of Exopolysaccharides

The enzymes involved in exopolysaccharide biosynthesis may be classified into four types: Group I, those enzymes which are involved in the initial metabolism of the substrate, such as hexokinases; Group II, enzymes responsible for the synthesis and interconversion of sugar nucleotides (examples include the enzymes UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase); Group III, transferases, which are enzymes responsible for the formation of the repeating monosaccharide unit attached to the carrier lipid; and Group IV, translocases or polymerases which form the exopolysaccharide biopolymer molecule. It has been postulated that each group of these enzymes is located in different regions of the microbial cell, thus having specific functions in the overall biosynthesis of the exopolysaccharide macromolecule.

The first group of enzymes are found intracellularly and are also involved in many other cell metabolic processes. The first of these enzymes, hexokinase, is involved in the phosphorylation of glucose (Glc) to glucose 6-phosphate (Glc-6-P) and then the second enzyme phosphoglucomutase converts Glc-6-P to Glc-1-P.

The second group of enzymes are believed to be intracellular. One of these enzymes, uridine diphosphate-glucose pyrophospho-

phorylase (UDP-glucose pyrophosphorylase), catalyzes the conversion of Glc-1-P to uridine diphosphate-glucose (UDP-Glc), which is a key sugar nucleotide and of great importance because UDP-Glc is the main precursor during exopolysaccharide biosynthesis. At this point it is important to emphasize the dual role sugar nucleotides such as UDP-Glc play in exopolysaccharide biosynthesis. The first role of UDP-Glc is its interconversion to sugar nucleotide UDP-galactose (UDP-Gal) with the enzyme UDP-galactose-4-epimerase, and/or its interconversion to the sugar nucleotide UDP-glucuronic acid (UDP-Glc UA) with the enzyme UDP-glucose dehydrogenase. The second role sugar nucleotides play is that they are donors of monosaccharide residues during the biosynthesis of the exopolysaccharide polymer molecule. The coenzyme uridine diphosphate (UDP) is an essential carrier of monosaccharide residues which become activated and transferred to suitable acceptors during polymer biosynthesis. However, all other homopolysaccharides require sugar nucleotides as donors of activated monosaccharide residues.

The third group of enzymes are located in the cell periplasmic membrane and are referred to as glycosyl transferases. They are involved in the transfer of sugar nucleotides, UDP-glucose or UDP-galactose and/or UDP-glucuronic acid to form the repeating unit attached to glycosyl carrier lipid present in the cell membrane. During this step the UDP is released while the sugar moiety is attached to the carrier lipid.

The fourth group of enzymes are the translocases or polymerases which are believed to be located on the outside part of the cell membrane and the cell wall. Although not much is known about these enzymes, it is presumed that they are involved in the polymerization of the macromolecule and then the exopolysaccharide is extruded from the cell surface to form a loose slime or a well attached polysaccharide capsule surrounding the cell. The exact mechanism of this 'extrusion' process of the exopolysaccharide macromolecules is not known.

Cell-Free Biosynthesis of Exopolysaccharides

The synthesis of the homopolymers, dextrans and levans, is catalyzed by extracellular enzymes acting on specific sugars, and therefore many of the constraints associated with a fermentation no longer apply to the design of a process for making these

polymers. For example, the synthesis of dextran using immobilized dextranase has been demonstrated and the use of cell free native dextranase is the basis of some commercial production. The synthesis of dextran by dextranase is well understood, for example controlled synthesis of dextrans of various molecular weights can be achieved by the altering enzyme and sucrose concentrations, temperature and the addition of small oligosaccharides or fractions of dextrans which act as primers.

In contrast to the homopolysaccharides, the synthesis of heteropolysaccharides involved several enzymes and cofactors thus making the commercial production of these polymers by cell free synthesis or immobilized cells less likely.

Processes for Microbial Exopolysaccharide Production

Processes for the production microbial exopolysaccharides are characterized by the extreme rheology of the fermentation broth, the low product concentrations at which this occurs and the diversity of subtle structural and conformational changes which can occur throughout the entire process and the marked effect of these changes on the product's end application performance. In the following section a description is given of how these general characteristics and more specific features of polysaccharides affect the design and operation of processes for their production.

Fermentation of Exopolysaccharides

The successful design of the fermentation stage of the process relies on producing a product to a set specification while achieving a product concentration, yield and productivity set from economic targets. These goals can best be reached with minimum risk by establishing how the microorganism's performance is controlled by its environment and, in turn, how this relates to equipment design operation (Figure. 27.4). The specific controls, which exist at these two levels, (i.e., environmental and equipment design and operation) and are characteristic of microbial exopolysaccharide fermentations, are described below.

Environmental control

Control of the composition of the fermentation medium, its addition and other environmental parameters are critical in achieving the desired rates of synthesis and yields of microbial

polysaccharides, and the complete utilization of the full heat and mass transfer capabilities, or energy input, of existing fermenters. These parameters are also prime determinants of the purity of the product, its chemical composition and molecular weight, which in turn determine its end use performance. Also, the strategy adopted in the running of the fermentation, e.g. batch draw fill, continuous, controlled feeding, etc., is also determined by the relative kinetics of cell and polymer production.

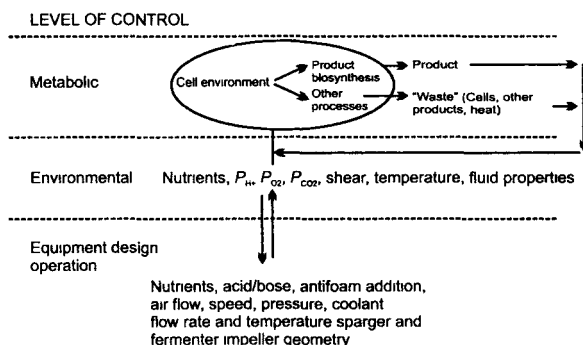


Fig. 27.4 : Levels of control governing microbial process

Fermentation Medium

The growth media which are suited to the production of different exopolysaccharides by microorganisms vary widely and this probably reflects the differing role of each polysaccharide in nature. For example, both xanthan produced by *Xanthomonas campestris* and alginate produced by *Azotobacter vinelandii* are made under carbon limited conditions whereas polymers produced by *Pseudomonas* NCIB11264 and *Klebsiella aerogenes* are not made under these conditions.

Effect of medium on specific rate of exopolysaccharide synthesis

Continuous culture studies on the effect of the growth limiting substrate on the synthesis of exopolysaccharides, by several different types of microorganisms, clearly demonstrate that the composition of the growth medium can dramatically affect the specific rate of polymer synthesis. Such effects are illustrated by the summary of some published data given in Table 27.3, and it is probable that in all the examples shown, the bacterial use the

activated sugar nucleotide pathway for polymer synthesis. It can be seen that for a given microorganism, although the polymer is produced under a variety of conditions, specific rates of synthesis can easily change by a factor of two or even cease under certain limitations. As expected, the absolute values of the specific rates appear to vary between species and strains, and also there appears to be no particular limitation which gives a common highest relative rate of production between organisms.

Table 27.3 : The Effect of Growth Limiting on the Specific Rate of Exopolysaccharide Production

<i>Microorganism</i>	<i>Polymer</i>	<i>Limiting nutrient</i>	<i>Specific rate of polymer production (gg⁻¹cells h⁻¹)</i>
<i>X. campestris</i>	Xanthan	Glucose	0.12
		NH ₄ ⁺	0.22
		PO ₄ ³⁻	0.09
<i>A. vinelandii</i>	Alginate	Sucrose	0.25
		N ₂	0.22
		PO ₄ ³⁻	0.28
<i>Ps. aeruginosa</i>	Alginate	Glucose	0.19
		N (yeast extract)	0.27
		NH ₄ ⁺	0.34
<i>Rhizobium</i>	Not identified	Mannitol-asparagine	0.0017
<i>Drifolli</i>		SO ₄ ²⁻	0.0049
<i>Pseudomonas</i>	Acetylated	Glucose	0
NCIB11264	polymer containing glucose, galactose, rhamnose and mannose	NH ₄ ⁺	Produced
<i>K. aerogenes</i>	Not identified	Glucose	0
		NH ₄ ⁺	Produced

Effect of medium on exopolysaccharide yield

The amount of (carbon) substrate converted by the cell to polymer depends on the composition of the growth medium and, under certain conditions, the product may not be made at all. Generally, media containing a high carbon to limiting nutrient ratio, often nitrogen, are favoured for polysaccharide production. Conversions of 70-80% of utilized glucose into crude polymer are

commonly found in high yielding polysaccharide fermentations. Care must, however, be taken in the interpretation of such yields as the crude product will often contain cells, other organic material and inorganic salts which are coprecipitated with the polymer when it is recovered from the fermentation broth. For example when corrected for the presence of contaminants, yields of 50-60% xanthan from glucose consumed can be obtained in well run processes, and this compares with a theoretical yield of about 0.85, with no cell growth, depending on the degree of pyruvulation of the polymer and the energy efficiency (P/O ratio) of the cell (Table 27.4). Some examples of the effect of medium composition on the yield of cell free polymer are given in Table 27.5.

Table 27.4 : Maximum Theoretical Yields of Xanthan^a at Different P/O Ratios

P/O ratio	Xanthan (g)	Xanthan (g)
	Glucose (g)	Oxygen (g)
1	0.81	5.9
2	0.86	10.7
3	0.87	12.5

^a Acid form of xanthan containing 1 mole of acetate and 0.5 mole pyruvate per repeating subunit.

Table 27.5 : The Effect of Growth Limiting Nutrient on Yield of Exopolysaccharide

Microorganism	Polymer	Limiting nutrient	Yield ^a
<i>X. campestris</i>	Xanthan	Glucose	0.54
		NH ₄ ⁺	0.60
		SO ₄ ²⁻	0.53
		Mg	0.55
		K ⁺	0.42
		PO ₄ ³⁻	0.31
<i>Ps. aeruginosa</i>	Alginate	Glucose	0.33
		N (yeast extract)	0.61
		NH ₄ ⁺	0.53

* Each set of results are from experiments conducted at constant dilution rate, and the value given is the amount of cell free polymer produced per unit of glucose consumed.

Effect of medium on exopolysaccharide composition

Manipulation of exopolysaccharide composition by changing the growth medium is one possible mechanism by which the properties of the polymer can be tailored to the end application. Experimental work on this aspect appears limited, but does indicate that although it may not be possible to alter the structure or

composition of the base repeating unit of the polysaccharide, it is possible to change the degree of substitution of the repeating unit by various groups and the degree of polymerization (molecular weight).

The properties result from its chemical structure (see Fig. 27.1), together with the various macromolecular forms of xanthan that can occur in solution (Fig. 27.5). Variation in the pyruvate content of the polymer tends not to change the polymer's gross characteristic properties, but it does affect the incremental changes in rheology which occur when the concentration of the polymer or the solution's ionic strength, pH or temperature are altered. These effects appear to be primarily associated with a promotion of the aggregated form of xanthan by pyruvate residues, whereas products which have a low pyruvate content tend to exist in disaggregated subunits. Solutions in which the aggregated form dominates exhibit high apparent viscosity, pseudoplasticity and yield stress. Thus control of the pyruvate content of xanthan, for example, through manipulation of the growth medium, represents a very useful way of fine tuning the properties of a polysaccharide.

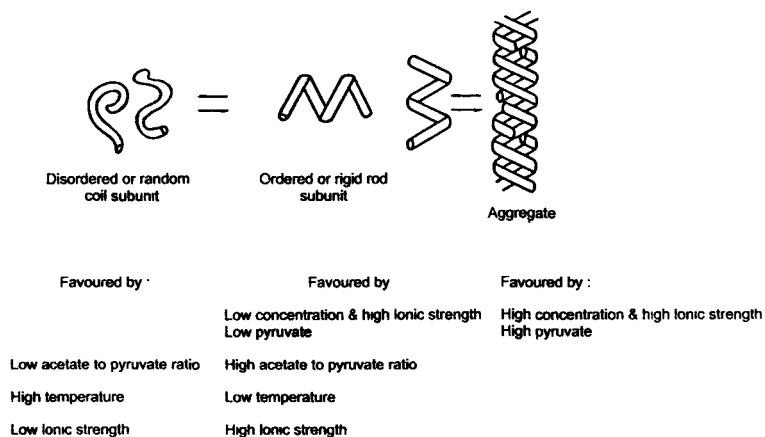


Fig. 27.5 : Conformational forms of xanthan in solutions.

Similarly, it has been shown that the acetate content of alginate is variable and appears associated only with mannuronic acid residues, and it has been proposed that acetylated mannuronic methods for controlling the acetate content of alginate and its influence on rheological properties are not well understood.

The reduction of the phosphate concentration to growth limiting levels in the culture medium for certain yeasts results in a shift from the production of type 11 phosphomannans, in which the terminal non-reducing end group is phosphorylated, to a structurally related mannan and it has been proposed that there is a common pathway for the two polymers in these yeasts. The concentration of phosphate in the polymer would be expected to markedly affect its rheological properties, through polyelectrolytic effects on the polymer's conformation.

Theoretical calculations show that the net energy required for polymer synthesis can vary from a positive to negative value in exopolymers containing oxidized groups, such as xanthan and alginate, as the content of these groups increases (Figure 27.6). These calculations account for the ATP used in polymerization and the ATP generated from the glucose used in polymer synthesis when metabolized via the operating pathways, i.e. the Entner-Doudoroff pathway, the TCA pathway and oxidative phosphorylation of the net reduced pyridine nucleotide produced. The results suggest that although these moieties are not essential for growth and do not affect the gross characteristics of the polymer, they may be important to the energy metabolism of the cell providing either a source or sink for energy when incorporated into the exopolysaccharide.

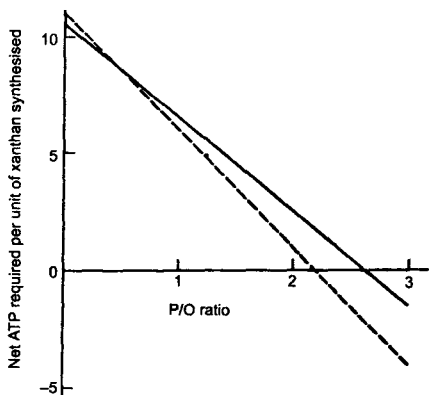


Fig. 27.6 : Net ATP requirements for synthesis of xanthan with various substituents. -----, 1 acetate + 1 pyruvate: ————, 1 lactate + 0 pyruvate

The molecular weight of a polymer has a direct influence on its rheological behaviour, with larger molecules having high viscosity.

Although it is known that the molecular weight of xanthan and other microbial exopolysaccharides can vary, little is known about chain termination and how the growth medium can be altered to control it. In some fermentations exocellular enzymes are secreted which degrade their own polymer. Suppression of the activity of the lytic enzyme, alginate lyase, in the *Azotobacter vinelandii* fermentation remains one of the major problems in economical production of high molecular weight microbial alginates. However, recently a possible solution has been suggested, being illustrated by the increase in viscosity that followed the addition of protease to a *Pseudomonas mendocina* and presumably resulted from proteolytic degradation of the alginate lyase. A similar problem is encountered in the pullulan fermentation in which there is a decline in the culture viscosity and molecular weight towards the latter stages of growth

In addition to the effects of medium composition on fermentation performance, as discussed above, other considerations must be taken into account. The composition of the medium can affect the nature of the recovery processing or the purity of the final product. For example, insoluble media solids either have to be removed prior to polysaccharide precipitation or they will be coprecipitated with the polymer. Also, high concentrations of inorganic salts in the medium can result in coprecipitates being formed when the polymer is isolated, giving the final product an increased ash content.

Other environmental parameters affecting exopolysaccharide fermentation

The growth and production of exopolysaccharides by microorganisms are determined by a wide range of environmental parameters, in addition to the effects of the culture medium described in the previous section. However, the influence on the fermentation of variables such as temperature, pH, dissolved oxygen and carbon dioxide, shear, etc., appears to have received less attention in published studies.

One early study reported the optimum temperature for xanthan production, as measured by culture viscosity, as 28°C and for growth 24 to 30°C. In a more detailed study, Williams studied the effect of temperature on the production of exopolysaccharide by *Pseudomonas* NCIB11264 under controlled conditions in continuous

culture. They found that in a steady state under nitrogen limited conditions, the cell concentration remained constant between 20 to 37.5°C, whereas the conversion efficiency of glucose to polymer, and culture viscosity, varied sharply with temperature showing a maximum at 30 °C. Interestingly, the polymer composition appeared unaffected by the fermentation temperature.

Early work on the xanthan fermentation demonstrated the beneficial effects of pH control on the production of charged polymers. Lack of pH control and poor medium buffering results in a rapid fall in the pH of the xanthan fermentation with a cessation of growth and production of the polymer at about pH 5.5. A pH optimum of 7 has been reported for the production of an acidic exopolysaccharide by *Xanthomonas campestris*, *Pseudomonas* NCIB11264 and other organisms. In the case of polymer production by *Pseudomonas* NCIB 11264 it was also shown that cell growth was less sensitive to pH than product synthesis and that the composition of the gum was unaffected by pH. The optimal pH for production of the neutral glucan, pullulan, by *Aureobasidium pullulans* was found to be about 5.3 and again was much more sensitive to pH than cell growth.

Oxygen is usually required in both the synthesis of components of the polymer (e.g. sugar acid) or indirectly in the oxidation of reduced pyridine nucleotides generated. Theoretical calculations for the synthesis of xanthan illustrate the amount of oxygen required and its dependence on the energy efficiency of the cell (Table 27.6). However, little information is available on the effect of dissolved oxygen tension and oxygen availability on growth and product formation in polysaccharide fermentations. Experiments and their interpretations are made difficult by the poor mixing often encountered in laboratory scale polysaccharide fermentations and the failure to independently separate the variables of oxygen tension and supply from shear and vessel homogeneity. In studies on alginate production by the nitrogen fixing obligate aerobe *Azotobacter vinelandii* it has been found that the conversion efficiency of sucrose to alginate increased with decreasing specific respiration rate in response to changes in oxygen supply. Under conditions of high dissolved oxygen tension the organism increases its respiration rate in an apparent attempt to protect its oxygen sensitive nitrogenase, with the resultant effect of diverting sucrose from polymer production to carbon dioxide.

Kinetics of Microbial Exopolysaccharide Synthesis

The relationships between growth and polysaccharide formation vary between microorganisms and for a particular fermentation they change with growth conditions and microbial strain. An interesting example is the specific rate of alginate synthesis in continuous culture, which is relatively independent of growth rate when produced by *Azotobacter vinelandii* but is strongly dependent upon growth rate when produced by *Pseudomonas aeruginosa*. Other examples are listed in Table 27.6

Table 27.6 : Kinetics of Polymer Formation by Microorganisms Under Various Conditions

<i>Microorganism polymer</i>	<i>Growth conditions</i>	<i>Kinetics of Polymer formation</i>
<i>Acinetobacter calcoaceticus</i> / rhamnose-glucose exopolymer	Batch	Mixed
<i>Zoogloea ramigera</i> / exopolymer	Batch, N limited	Non-growth associated
<i>Alcaligenes faecalis</i> / curdlan	Batch, N limited	Non-growth associated
<i>Pseudomonas aeruginosa</i> / alginate	Batch, and continuous N limited	Growth associated
<i>Alcaligenes vinelandii</i> / alginate (parent strain) (Mutant)	Batch, P limitation continuous, P limitation	Growth associated Non-growth associated ^a
<i>Rhizobium trifolii</i> / exopolymer	Batch, P limitation continuous, mannitol, asparagine limited	Mixed Growth associated
<i>Pseudomonas</i> NCIB11264/ exopolymer	Batch and continuous, N limited	Non-growth associated ^a
<i>Xanthomonas campestris</i> / xanthan	Batch and continuous, N limited	Mixed
<i>X. juglandis</i> /xanthan	Continuous, S limited	Non-growth associated ^a

^a Non growth associated production in continuous culture occurs when the specific rate of polymer formation is independent of growth rate.

In growth associated fermentations, a high rate continuous process is best suited to high rates of polysaccharide production. Whereas with non-growth associated products, the best process

will be a batch process in which there is a rapid period of cell growth followed by a stationary phase (or by greatly reduced growth achieved by controlled feeding of a limiting nutrient), during which time the product is generated. Draw fill techniques may also be applied to the batch process to increase overall fermenter productivity.

Table 27.7 : Rheological Properties of Some Polysaccharide Fermentations

<i>Rheological property</i>	<i>Description</i>	<i>Examples of fermentations showing relations</i>
Pseudoplastic flow or shear thinning	Apparent viscosity of the fluid decreases with increasing shear rate	Pullulan (Leduy et al., 1974), xanthan (Jeanes, 1974; Pace and Righelato, 1980), zoogloea ramiger (Norberg and Enfors, 1982, mannans and Phosphomannans (slodki <i>et al.</i> , 1974), alginate (Lawson and Sutherland, 1978)
Yield stress	A given stress has to be applied to the fluid before movement occurs	Xanthan (Jeanes, 1974; Solomon, 1980)
Thixotropy	Apparent viscosity of a Pseudoplastic fluid decreases with time at a constant shear rate	Alginate fermentations containing high levels of calcium.
Viscoelasticity	Combinations of viscous and elastic behaviour. This can result in such effect as the fluid having a'memory' (a tendency to regain its original state on release of a stress); normal stress which results in the stirrer climbing or Weissenberg effect	Xanthan (Solomon, 1980)

Fermenter Design and Operation

An understanding of the relationships between the environment in a fermentation and the equipment and operating variables is the key to the design and scale up of equipment for economic production. In microbial exopolysaccharide fermentations, the extreme rheology encountered has the major

influence on the nature of these relationships compared to other low viscosity fermentations, such as bacterial and yeast cultures. The picture is further complicated in that changes in heat, mass and momentum transfer caused by increases in culture rheology can feed back to further effect either the polymer's rheological behaviour or the ability of the microorganism to produce the polymer.

The major contributor to the rheology of exopolysaccharide culture fluids is the polymer dissolved in the continuous phase, which contrasts with filamentous fungal fermentations in which the high viscosity is caused by mycelium or the discontinuous phase. This can lead to differences in the heat and mass transfer achieved in experiments conducted on mycelial and polysaccharide fermentation at equivalent measured rheologies on the same equipment.

The rheology exhibited by microbial polysaccharide culture fluids is complex and includes pseudoplastic flow behaviour (which is commonly observed), thixotropy, yield stress and viscoelasticity. These terms are described in Table 27.7.

Mixing Characteristics

The flow patterns and mixing in agitated vessels are markedly affected by the fluid's rheology. Highly viscous pseudoplastic fluids show good movement in the region of the impeller where shear rates are high (Figure 27.7). However, away from the impeller the movement decreases due to the apparent increase in viscosity resulting from the pseudoplastic nature of the fluid. High viscosity also decreases the pumping capacity of the impeller which further compounds the problem. Thus these effects promote gas funnelling through the centre of the vessel and inhomogeneity, particularly in areas away from the impeller near the vessel wall, and virtually static areas may form around baffles and in small vessels around probes. The tendency to form dead zones is further enhanced if the fluid possesses a yield stress. As shown in Figure 27.7, in highly viscous pseudoplastic fluids, particularly if they have a yield stress (e.g. xanthan), a well mixed region, termed a cavern, can exist around the impeller with no movement in the surrounding fluid. The size of this cavern for both aerated and unaerated conditions has been measured and a model for predicting its size developed and such an approach may be helpful in predicting the conditions necessary for good mixing. The blending time in vessels,

particularly those containing highly viscous fluids, increased with gas hold up. For impeller Reynolds number in the transition regime viscoelastic fluids show reversed flow patterns compared to elastic fluid. Although flow reversal is unlikely to occur in the impeller region in a polysaccharide fermentation, such effects may be important in determining local mixing at some distance from the impeller (Figure 27.7).

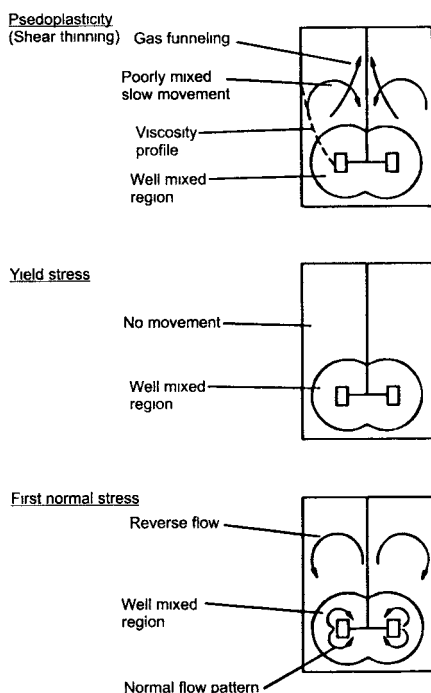


Fig. 27.7 : The effects of various forms of rheological behaviour on flow patterns in agitated vessels.

Fermenter Design

It is generally agreed that a mechanically agitated fermenter is required to achieve good heat and mass transfer at high viscosities.

At extreme viscosities, the pumping capacity of the standard flat bladed turbine impellers falls dramatically and results in a poorly mixed fermenter capable only of low rates of heat and mass transfer.

On the other hand, in low viscosity fermentations this type of impeller appears to give adequate mixing and in most cases this does not limit heat and mass transfer. Thus the design of the agitation system for use in polysaccharide fermentations requires special attention to give the correct distribution of power to ensure good culture homogeneity and turbulence, to minimize bubble coalescence, to promote small bubble formation and to achieve adequate fluid movement at heat transfer surfaces, and will inevitably, require non-geometric scale up.

Improvements over the flat bladed turbine include the use of backward swept turbines for intermediate viscosity fluids or the use of two different types of impeller, one for mass transfer (small bubble generation) and one type for mixing (promotion of bulk flow) at higher viscosities. Through the use of flow visualization techniques, Solomon has shown that the power required to mix fully a small vessel is markedly decreased when a combination of a flat bladed turbine, and a 45° axial flow impeller vs, a two flat bladed impeller of the same diameter, are used to agitate high viscous pseudoplastic fluids. In order to minimize the power input to achieve the desired rates of heat, mass and momentum transfer into a fermenter used for viscous mycelial fermentation, Anderson used two separate drives for the gassing dispersing turbine impeller and the slower moving bulk flow promoting axial flow propeller.

Recovery of the Exopolysaccharide Product

The cost of recovery of microbial polysaccharides, including concentration, isolation and purification, is a significant part of the total production cost and is due to the dilute nature of the stream leaving the fermenter (about 15 to 30 kg m⁻³), the presence of contaminating solids (e.g. cells) and solutes in the stream and its high viscosity. The objectives of the recovery processing are (i) concentration of the fermentation broth or extract to a form, usually a solid which is microbiologically stable, easy to handle, transport and store and can be readily redissolved or diluted for use in a particular application; (ii) purification to reduce the level of non-polymer solids, such as cells or salts, and to improve the functional performance, colour, odour or taste of the product; (iii) deactivation of undesirable contaminating enzymes, such as cellulases, pectinases, etc., and (iv) modification of the chemical properties of the polymer to alter either the functional performance, the solid's

handling properties of the dried product or the dispersion and solution rate characteristics.

Two examples of recovery processes which have been suggested for the production of microbial alginate and xanthan are shown in Figure 27.8, and in general the processing steps are similar to those used in the harvesting of plant and algal gums. In the following sections the commonly described operations are discussed and further information can be found elsewhere.

Cell Removal

The presence of cells in the final product can directly affects its end application performance as well as diluting the amount of polymer present. Cell removal may be essential, for example, if the gum is to be used in a product which is optically clear, such as certain cosmetics or foods, or in enhanced oil recovery where the presence of cells in the polymer flood fluid causes plugging of the pores of the oil bearing rock.

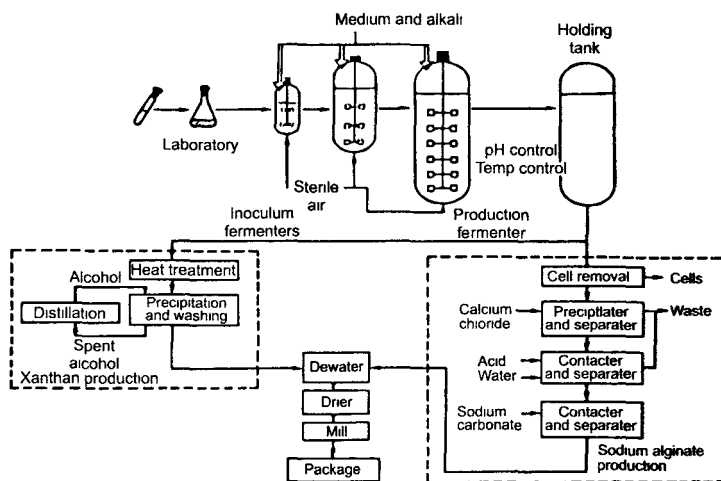


Fig. 27.8 : Processes for the production of the microbial polysaccharides xanthan and alginate.

The high viscosity of a typical polysaccharide fermentation broth results in conventional cell separation steps such as centrifugation, filtration and flocculation being expensive, and to give practical rates of processing it is often necessary to dilute the

broth. Also, some advantage may be derived during processing by heating the broth to lower its viscosity, however care must be taken not to degrade the product and, in the case of xanthan, the fluid must be heated to more than 100°C before the viscosity falls.

Isolation of Exopolysaccharide

The isolation of a polysaccharide is achieved by lowering of the solubility of the polymer to result in either a precipitate or a phase consisting of a concentrated solution, a coacervate, by either the addition of a water miscible solvent, e.g. methanol, ethanol, isopropanol, acetone (note that in strict terms it is a non-solvent for the polymer), or salt or acid. The molecular mechanisms behind these methods are described but broadly rely on a decrease in the polymer-solvent (water) affinity by either changing the net charge on the polymer (addition of salt or an acid) and/or through competition with the polymer for water (addition of large amounts of salt), or by changing the hydrophilicity of the solvent (addition of alcohol). The method of choice for isolation of the polysaccharide is determined by economics, practicability and the final specification of the product, with alcohol precipitation being widely favoured. Some examples of alcohol, salt and acid precipitation are given in Table 27.8.

The volume of alcohol required for precipitation of xanthan is relatively independent of polymer concentration but is affected by the concentration of certain salts. For example, addition of potassium chloride to xanthan broths decreases the amount of isopropanol required for precipitation by about 30%. Thus increasing the concentration of product during fermentation and increasing the broth's salt content prior to precipitation will decrease the amount of alcohol processed per unit weight of polymer recovered. Such improvements can significantly affect production economics. The coprecipitation of cells, certain inorganic salts and colour pigments with the polymer leads to decreased product purity and, hence, rheological performance or appearance and washing with alcohol/water will only partly remove such impurities. Also, adjustments in the fermentation medium and conditions can also be used to minimize coprecipitation and improve final product purity.

Table 27.8 : Examples of Methods used for the Precipitation of Microbial Polysaccharides.

<i>Method</i>	<i>Example</i>	<i>Comments</i>
Alcohol precipitation	Isopropanol	45-60% (w/w) of isopropanol required for xanthan cultures; precipitation conditions and washing can affect subsequent processing and final product (Bouniot, 1976; Roche, 1981)
Salt precipitation	Calcium hydroxide	Insoluble salt of alginate or xanthan formed; required acid titration to form soluble salt of polymers (Mehlretter, 1965; see Figure 27.8). Suitable for most polyanionic polysaccharides
	Quaternary ammonium compounds	Precipitate washed with methanol to give soluble salt (Albrecht et al., 1965); unlikely to be acceptable for a food grade process
Acid precipitation	Lower pH with hydrochloric acid	Alginic acid precipitated at pH<4; used in some commercial processes for extraction of algal alginate

Dewatering and Drying

The final purity of the product and the cost of drying can be improved if the precipitate is dewatered prior to drying, for example by pressing or centrifugation. Exposure of the product to excessive heat or mechanical stresses during dewatering can lead to product degradation.

Forced air or vacuum continuous or batch driers are normally used to dry the dewatered precipitate. The main aim of drying is to lower the water content of the product to a level where it is microbiologically stable and in an easily handled form. Drying conditions can also affect other properties of the polymer including its colour, solubility, rheological properties and solvent content. High drying temperatures can lead to poor colour and solubility, and product degradation.

An alternative approach to the recovery of the product is drying of the whole culture fluid. However, the product obtained contains a high level of impurities and colour and as such is not commercially desirable.

Milling and Packaging

The particle size of the dried product is controlled to ensure a proper balance between dispersability, i.e. the ability of the polymer to be dispersed in water without clumping, and dissolution rate, i.e. the rate at which it dissolves into solution. For example, large particles tend to be more readily dispersed but slower dissolving compared to smaller particles, which tend to enter solution quickly but are more difficult to disperse. Controls of these properties is important to the end user as it determines the method and type of equipment required for making up solutions. Again excessive heating or mechanical stresses during milling can lower product quality.

Owing to the hygroscopic nature of the dried polysaccharide it is important that the polymer be packaged in containers with a low permeability to water. Moisture pick-up can lead to clumping and subsequent problems in solution make-up and in some cases hydrolytic degradation of the product.

Additional Processing

Gums may be physically or chemically treated or modified during recovery to affect their purity, cosmetic appearance, handling characteristics, rheological or other physical properties and the processing method used for a given polymer depends on its physical structure and chemical reactivity. Some specific examples of the types of changes that can be carried out on a microbial gum, xanthan, are given below.

Xanthan is an unusual polymer in that when solutions of high concentration containing salt are heated the viscosity remains approximately constant to temperatures in excess of 100°C. In addition to this phenomenon, controlled heating of xanthan fermentation broth in the region of 100°C for a few minutes results in a final dried product with enhanced low concentration and shear rheology owing to conformational changes or cross-linking of polymer chains. Heating of most other polymers at this temperature results in degradation, reflected in the low viscosity of the finished

product, and only when xanthan is heated above about 150 °C does it degrade rapidly. The dispersability of xanthan can be improved by reacting it with a dialdehyde such as glyoxal. When the dried glyoxal xanthan complex is added to water its low solubility aids its dispersion and as the glyoxal-xanthan bonds hydrolyse the polymer enters the solution.

Microbial exopolysaccharides may contain extraneous enzymes which restrict the range of materials with which the gum is compatible. For example, xanthan directly precipitated from culture fluid contains cellulase which is very active against other cellulosic gums, such as carboxymethylcellulose (CMC), and thus cannot be used together with CMC in an end application. However, heating or treatment of xanthan with an oxidizing agent such as propylene oxide may be used to inactivate the cellulase and render it compatible with CMC.

Polysaccharides can also be reacted with various chemicals to modify their structure and hence their rheological properties. Xanthan can be deacetylated by heating the gum under alkaline conditions, and the viscosities of solutions of the resultant product are claimed to be enhanced by the addition of salt. Cross-linking xanthan with formaldehyde gives a stable, water soluble form with enhanced viscosity. A graft polymer between polyacrylamide and xanthan can be formed and is claimed to have superior properties compared to xanthan when used as an additive in polymer floods in enhanced recovery.

Conclusions

Microbial exopolysaccharides are water soluble biopolymers produced by a wide variety of microorganisms grown on different carbohydrate and other substrates under submerged culture conditions. In view of their unique physical and chemical properties, exopolysaccharides have found a wide range of important applications in the pharmaceutical, food, chemical and other industries. The rheological characteristics of exopolysaccharides are of particular importance and this resulted in their use as emulsifiers, gelling agents, stabilizers, coagulants, binders, film formers, suspending agents and lubricants.

The structure and composition of microbial exopolysaccharides depends on many different factors, which may include medium composition, type of carbon and energy source, microbial system

used, and other fermentation conditions such as pH, temperature and oxygen concentration. Despite the commercial importance of microorganisms which produce exopolysaccharides, very little is known about the biochemistry of these microbial systems. A good understanding of the biochemical pathways is important because this information can be used to optimize and control the biosynthesis of these exopolysaccharides during fermentation. More research is needed to elucidate the various biochemical pathways involved in different exopolysaccharide systems.

Depending on the microbial/system used and fermentation conditions, the kinetics of exopolysaccharide production was found to be cell growth or non-growth associated or mixed. This diversity in the kinetics of different systems necessitates the choice of the best suited modes of fermentation, i.e. continuous, batch or semi-batch, in order to maximize the rate of exopolysaccharide production. During the course of fermentation, the broth starts as a low viscosity Newtonian system and ends up as a highly viscous non-Newtonian system as the exopolysaccharide concentration increases with time. These extreme rheological changes have profound effects on the mixing, mass transfer and heat transfer characteristics of exopolysaccharide fermentations. Optimal fermenter design and operation must take into account these rheological changes. A lot of work remains to be done on mixing, mass transfer and especially heat transfer characteristics of exopolysaccharide fermentations.

A process economic feasibility study revealed that the cost of downstream recovery and purification of the exopolysaccharide product represents a significant part of the total production cost. Very little information is available in the research literature on the optimization of recovery and purification of exopolysaccharides. Microbial exopolysaccharides represent only a small portion of the total polysaccharide market, most notable of which are xanthan gum and dextrans. At the present time the production cost of microbial polysaccharides is higher than the cost of other traditional polysaccharides, such as cornstarch and cellulose-derived products, which dominate the market. However, the competitive advantage of microbial polysaccharides will improve as more research and development work will undoubtedly result in lower production costs, and new industrial speciality applications will create higher demand for these products.

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